STUDIES ON RAT HEPATIC BILE ACID-BINDING PROTEINS USING PHOTOAFFINITY LABELLING TECHNIQUES

by

COLIN J HENDERSON, B.Sc.(Edin.)

A thesis submitted for the degree of
Doctor of Philosophy, University of Edinburgh

1984

DECLARATION OF ORIGINALITY

I declare that the work presented herein and the composition of this thesis are my own.

Colin J Henderson

ACKNOWLEDGEMENTS

I am most grateful to my supervisors Dr J D Hayes, Professor L G Whitby and Professor I W Percy-Robb for their advice, support and interest throughout this project. Professor L G Whitby is thanked for the use of laboratory facilities in the Department of Clinical Chemistry, University of Edinburgh.

I thank Dr J D Hayes for the gift of purified glutathione S-transferases, Dr I Gosney, Department of Chemistry, University of Edinburgh, for the ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, and Dr J E T Corrie, Teaching and Research Unit, Western General Hospital, Edinburgh, for advice concerning the synthesis of the photoaffinity label.

I also thank Mrs E Ward for typing this thesis and the Medical Illustration Department, University of Edinburgh, for the artwork. Finally, I wish to acknowledge the University of Edinburgh for a Faculty of Medicine Scholarship which enabled this work to be carried out.

I would like to dedicate this thesis to my family, and in particular, to my wife Bridget in grateful recognition of her constant support and enthusiasm.

ABSTRACT

The transcellular route that bile acids follow during the hepatic phase of the enterohepatic circulation has been a matter of controversy for some time. Among the modes of intracellular transport proposed have been free diffusion and partitioning within cellular organelles, vesicular transport, and association with cytosolic binding proteins.

It is known that several groups of bile acid-binding proteins exist in rat hepatic cytosol; these include Z protein(s) and the glutathione S-transferases (Y proteins). A new group, the Y' proteins, has been discovered recently and a purification scheme reported for two bile acid-binding proteins, which have been partially characterised.

The aims of this study have been to investigate bile acid-binding proteins from rat hepatic cytosol, with particular reference to the Y' proteins. The technique of photoaffinity labelling has been employed as a method of assessing the bile acid-binding potential of these proteins. The photoaffinity label used in these experiments has been [^{125}I]-3βazidocholylhistamine, a photolabile derivative of cholic acid, radioiodinated to a specific radioactivity of 1900 Ci/mmol. This labelled molecule was shown to retain the properties of the parent bile acid with regard to hepatic transport.

In photoaffinity labelling studies with cytosolic proteins from rat liver, previously undescribed bile acid-binding proteins have been identified in the Y' fraction. These proteins were purified using gel filtration, ion-exchange, chromatofocusing and hydroxyapatite chromatography. The resulting homogeneous proteins fell into two categories:-

1) Proteins (designated binders 5B, 6E and 7F) consisting of two identical subunits each having an approximate molecular mass of 15 000 - 19 000 Da. These showed a high specific incorporation of radioactivity after photoaffinity labelling, and were present at low concentrations.

2) Proteins (designated binders 5C, 5D and 8C) of molecular mass approximately 33 000 - 36 000 Da. These showed a lower specific incorporation of radioactivity after photoaffinity labelling than those in group 1, but had a higher total incorporation of [^{125}I] owing to their higher concentration in the liver.

These proteins were analysed by peptide "mapping" on reverse-phase high performance liquid chromatography to determine their genetic relationships. In addition, along with several glutathione S-transferases, they were photoaffinity labelled and peptide "mapped" to determine the position of the radioactive label, and thus the bile acid-binding site, within the molecule.

INDEX

FIGURES AND TABLES

INTRODUCTION

STRUCTURE AND PHYSIOLOGY OF BILE ACIDS

Bile acid structure

The molecular structure of bile acids is based on perhydrocyclopentanophenanthrene, a nucleus of four rings, labelled A to D. Figure 1.1 shows the prototype bile acid, cholanic acid. The number and arrangement of hydroxyl groups around this basic molecule varies with individual bile acids - for example, cholic acid has hydroxyl groups at positions 3, 7 and 12, chenodeoxycholic acid at 3 and 7, deoxycholic acid at 3 and 12, and lithocholic acid a single hydroxyl at position 3. The molecule is not flat; rather it is L-shaped, bent at the A-B ring junction, with the hydroxyl groups and carboxyl sidechain oriented to one side of the molecule (α-orientation) and the methyl groups to the other side (β-orientation). This arrangement has the effect of dividing the molecule - one side is polar (hydroxyl groups and carboxyl sidechain), while the other is non-polar or hydrophobic (ring nucleus and methyl groups). A perspective formula of deoxycholic acid is also shown in Figure 1.1.

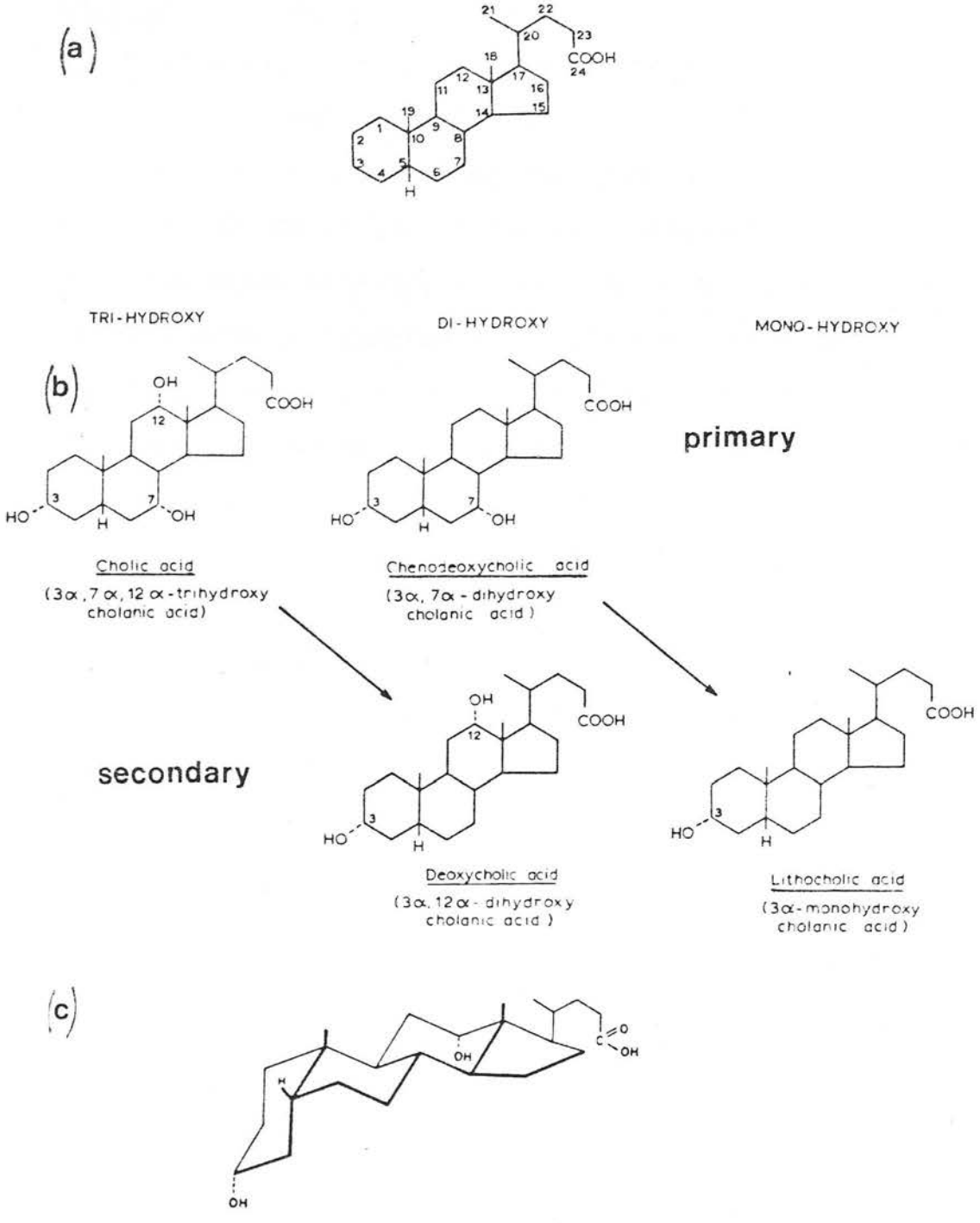


FIGURE 1.1 Bile acid structure

(a) cholanic acid, with C atoms numbered;

(b) primary and secondary bile acids;

(c) perspective formula of deoxycholic acid;

Bile acid function

Bile acids have three physiological roles:-

1) Emulsification of fat

2) Stimulation of bile flow

3) Excretion of excess cholesterol

1) Bile acids emulsify dietary lipid in the intestine, thereby aiding digestion and subsequent absorption (Hofmann & Borgstrom, 1962; Hofmann, 1963, 1976). That they are able to do this is due to their amphipathic properties - that is, in common with other detergents, one part of the molecule is non-polar and water insoluble, and the other is polar and soluble. When the concentration of bile acids reaches a certain level, the critical micellar concentration (0.75 - 2 mM), micelles are formed; these are polymolecular aggregates of bile acids about 10 nm in diameter, discoid in shape and comprising 4-20 bile acid molecules (Holt, 1972). Each molecule is oriented with the hydrophobic areas towards the centre of the aggregrate and the polar groups on the outside directed towards the aqueous phase (Hofmann & Small, 1967; Carey & Small, 1972).

Most dietary lipid is in the form of triglyceride, the digestion of which is catalysed by pancreatic lipase, acting on the fat droplets in the intestine. Bile acids assist by emulsifying these droplets, thereby increasing the effective surface area available on which the enzyme acts. The triglycerides are broken down into mono-

glycerides and fatty acids, which are then solubilized by bile acids in the form of mixed micelles (Small, 1970). In such micelles, where the digestion products are intermingled with the bile acid molecules, the monoglycerides and fatty acids are delivered to the intestinal mucosal surface where they are absorbed, at a rate apparently directly related to their concentration in micellar solution (Simmonds, 1974). The fat-soluble vitamins, which are very poorly soluble in water, are also handled in the same manner, and biliary cholesterol, too, is solubilized in this way (Admirand & Small, 1968; Neiderhiser & Roth, 1968; Dam & Hegardt, 1971; Ockner & Isselbacher, 1974).

2) Bile acids are also believed to have choleretic activity. This idea is based on observations that the active transport of any compound, including bile salts, into bile, would result in water and other solutes following due to osmotic effects; thus bile flow would be stimulated (Sperber, 1963, 1965). The portion of canalicular bile flow that results from bile acid excretion is termed bile acid-dependent flow (Boyer & Klatskin, 1970; Wheeler, 1972; Boyer & Bloomer, 1974). The relationship between these two processes was believed to be linear, but recent work has shown a curvilinear variation; this is thought to be due to bile acids forming micelles at higher concentrations and thus being less osmotically potent (Balabaud et al., 1977; Baker et al., 1979; Blitzer & Boyer, 1982).

3) Bile acids also provide a means whereby the body may excrete excess cholesterol; elevated serum cholesterol levels are associated with arterial disease (Elliot & Hyde, 1971). Indeed, bile acid synthesis is believed to play an important role in cholesterol homeostasis (Danielsson & Sjovall, 1975). Cholesterol is both present in the diet and synthesized endogenously. Bile acids are synthesized from cholesterol in the liver (for details see p.8-9) and secreted into bile with cholesterol, where they form mixed micelles. Ultimately, the cholesterol is excreted in the faeces whilst the majority of the bile acids are recycled to the liver to further mediate cholesterol excretion. Thus, bile acids are not only quantitatively the major products of cholesterol catabolism, but also function in solubilizing and helping to eliminate their own precursor from the body (Myant & Mitropoulis, 1977).

Bile acids may also regulate their own synthesis, and that of cholesterol, through negative feedback inhibition (Myant & Mitropoulis, 1977). The rate of bile acid synthesis is apparently controlled by the size, composition and circulation rate of the bile acid pool returning to the liver (Shefer, 1969, 1970; Danielsson, 1973). In experiments where the enterohepatic circulation (for details, see p.15) is interrupted either by ileal resection, or by oral administration of the bile acid-binding resin cholestyramine, or by external

diversion of the bile, the rate of bile acid synthesis is increased, as indeed is the rate of cholesterol synthesis (Shefer et al., 1968, 1972; Boyd et al., 1969; Back et al., 1969; Grundy, 1977). Conversely, the oral administration of bile acid leads to a decrease in the rate of synthesis (Einarsson et al., 1973; Schoenfield et al., 1973; Shefer et al., 1973a,b; Danielsson & Johanssen, 1974). Although the site of action of bile acids in the regulation of both their own synthesis and of cholesterol is unclear, there is evidence to suggest that the hepatic bile acid concentration may control the activities of two enzymes, cholesterol 7α-hydroxylase and 3-hydroxy-3-methyl glutaryl CoA (HMG CoA) reductase (Mosbach & Salen, 1974). The former catalyses the first step in bile acid synthesis, and is rate-limiting in both man and the rat, while the latter is known to be rate-limiting for cholesterol synthesis (Einarsson et al., 1973; Botham et al., 1979). However, more recent work with cultured rat hepatocytes has shown that bile acids have no direct effect on bile acid synthesis by a feedback mechanism (Botham et al., 1980, 1981). In addition, Davis et al. (1983) speculate that a factor derived from the intestine, which would be absent in culture, may initiate negative feedback control. The observation that there appears to be a concerted regulation of 7α-hydroxylase and HMG CoA reductase may be explained by cholesterol availability as a function of HMG CoA reductase regulating bile acid synthesis (Cronholm &

Sjovall, 1967; Danielsson, 1972; Myant & Mitropoulis, 1977; Davis et al., 1983b). Alternatively, a common factor may regulate the synthesis of both bile acids and cholesterol (Davis et al., 1983a).

Bile acid synthesis

The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized only in the liver from cholesterol (Elliot & Hyde, 1971; Boyd & Percy-Robb, 1971; Danielsson, 1973; Bjorkham & Danielsson, 1974). Studies in vivo and in vitro have indicated that the synthetic pathways involved are the same or similar in man and the rat (Daniellson & Sjovall, 1975). For both bile acids, the first step is the introduction of a hydroxyl group into the 7α position by the microsomal enzyme 7α -hydroxylase, requiring NADPH, molecular oxygen and cytochrome P450 for activity (Danielsson & Sjovall, 1973; Shefer et al., 1975). It is currently believed that this step is rate-limiting for bile acid synthesis (Myant & Mitropoulis, 1977). The 3β-hydroxy group of 7α-hydroxycholesterol is oxidized to a 3-keto group, and the Δ^5 double bond is isomerised to the Δ^4 position by other microsomal enzymes to give 7α-hydroxy-4-cholesten-3-one (Mendelsohn et al., 1966; Bjorkhem & Daniellson, 1967). This compound represents a branch point in the synthesis of the two principal primary bile acids. Within the endoplasmic reticulum, a hydroxyl group is inserted at the 12α position by microsomal 12α-hydroxylase and the

synthesis is committed to cholic acid (Danielsson & Einarsson, 1966; Bjorkhem & Danielsson, 1967; Percy-Robb, 1975). Alternatively 7α-hydroxy-4-cholesten-3-one is passed into the cytosol where it is converted ultimately into chenodeoxycholic acid (Percy-Robb, 1975). With both reactions, modifications to the ring structure occur first, followed by alterations to the sidechain. Final conversion occurs within the mitochondria, involving hydroxylation at C-26 (Matern & Gerok, 1979).

An alternative pathway apparently exists in mitochondria, which is initiated by hydroxylation of C-26 in the cholesterol sidechain (Danielsson, 1973; Taniguchi, 1973; Danielsson & Sjovall, 1975). Both cholic acid and chenodeoxycholic acid can be synthesized in this manner (Anderson et al., 1972). However, difficulty with subsequent insertion of the 12α-hydroxyl group after sidechain hydroxylation means that the predominant product is chenodeoxycholic acid (Hansen, 1971). Small amounts of cholesterol may also be converted through this pathway into lithocholic acid, which may have toxic properties (Javitt, 1975), (see p.14).

Bile acid metabolism

Following their synthesis from cholesterol, bile acids are metabolized, both in the liver before being excreted into bile, and also in the intestine following excretion. The following mechanisms are involved in the metabolism of bile acids:-

1) Conjugation - with either glycine or taurine;

2) Glucuronidation;

3) Sulphation;

4) Dehydroxylation and deconjugation.

1) Conjugation

Glycine and taurine are the two major amino acids which are linked to the carboxyl group of the bile acid sidechain, both in humans, where the ratio of glycine to taurine conjugates is 3:1 under normal conditions, and also in the rat where the ratio is 20:1 (Sjovall, 1960). In both cases the ratios can be altered by nutritional factors or disease states (Garbutt & Kennedy, 1972). The conjugation reaction takes place in two steps (Elliot, 1955; Bremner, 1956). The first is the ATP-dependent formation of a CoA-bile acid derivative (Polokoff & Bell, 1977; Killenberg, 1978; Vessey & Zakim, 1977). This derivative is then reacted with glycine or taurine (Vessey et al., 1977). Whereas the rat possesses a single enzyme for the latter reaction, in humans there may be more than one bile acid-CoA:amino acid N-acyl transferase involved in the conjugation (Abberger et al., 1978; Killenberg & Jordan, 1978). Conjugation results in the pKa of bile acids being lowered; with glycine, the reduction is from 6 to 4 whilst with taurine, the pKa is reduced from 6 to 2 (Scharschmidt, 1983). As a result, at the pH of the intestinal lumen (approximately 6.5), the

conjugated bile acids are largely ionised and are more easily absorbed from the intestine (Schiff et al., 1972). In addition, the conjugated bile acids, being more polar, are excreted into bile more rapidly than their unconjugated counterparts (O'Maille et al., 1965). Moreover, conjugated bile acids are apparently less toxic to the intestinal mucosa (Pope et al., 1966; Teem & Phillips, 1972).

2) Sulphation

The 3α-hydroxyl group of the bile acid molecule can react with sulphuric acid and form a sulphate ester (Palmer, 1967). An enzyme which catalyses bile acid-sulphate formation has been found in human and rat liver, and also in rat kidney (Loof & Wengle, 1978; Chen et al., 1977). This enzyme utilizes 3'phosphoadenosine 5'-phosphosulphate (PAPS) as a cofactor and sulphate donor in the sulphation reaction (Chen et al., 1977).

The sulphate esters are of particular importance in the metabolism of the monohydroxy bile acids such as lithocholic acid. In man, this bile acid is preferentially sulphated, and it appears that this reaction reduces the intestinal absorption of such derivatives of glyco- and taurolithocholic acids, thus protecting the body from their potentially toxic effects (Cowen et al., 1975a, b).

3) Glucuronidation

A liver microsomal enzyme, UDP-glucuronyl transferase, will catalyse the transfer of a glucuronyl moiety from glucuronic acid to the 3β-position of the bile acid molecule. Such bile acid-glucuronides have recently been found in patients with cholestasis; 7-16% of the bile acids in the urine were glucuronidated (Back et al., 1974; Back, 1975; Frohling & Stiehl, 1976; Frohling et al., 1977). It is speculated that although glucuronidation of bile acids occurs quantitatively to a lesser extent than sulphation, the former may also be involved in the elimination of toxic monohydroxy bile acids by forming highly polar metabolites which are more water-soluble (Javitt & Emerman, 1968; Back, 1976; Alme et al., 1977).

4) Dehydroxylation and deconjugation

Breakdown of bile salts by bacterial action occurs mainly in the large intestine, but also to a smaller extent in the small intestine (Gustafssen & Norman, 1962; Aries & Hill, 1970; Tabaqchali, 1970). Bile salts are broken down by obligate anaerobes; a total bacterial count of over $10^4/mm^3$ is apparently required for bile salt breakdown to be significant (Drasar & Shiner, 1969; Gorbach & Tabaqchali, 1969). The low amount of bacteria present in the small intestine under normal physiological circumstances means that little bile salt breakdown occurs, except in the terminal part of the ileum, proximal to the ileocaecal sphincter, where anaerobes are found in

large numbers (Gorbach et al., 1967). In normal human subjects, only this area of the small intestine is found to have bacterially altered bile acids. However, as the rat eats its own faeces, it normally possesses a more extensive bacterial colonisation of the small gut.

The major changes brought about by intestinal bacterial action on bile salts are deconjugation and 7α--dehydroxylation (Lewis & Gorbach, 1972). However, oxidation, reduction and epimerisation can also occur (Matern & Gerok, 1979). Liberation of the free bile acid from the parent bile salt by deconjugation is effected by a cholanyl glycine hydrolase, produced by several strains of bacteria including Streptococcus faecalis and Clostridium perfringens (Aries & Hill, 1970; Lewis & Gorbach, 1972). This enzyme will also deconjugate other bile salts (Drasar et al., 1966; Dickenson et al., 1971).

The two primary bile acids, cholic acid and chenodeoxycholic acid, can be dehydroxylated at the 7 position to form the secondary bile acids, deoxycholic acid and lithocholic acid respectively (Lewis & Gorbach, 1972). Although deconjugation normally occurs first, 7α--dehydroxylation can take place without preceding deconjugation (Norman, 1970; Hepner et al., 1972a,b). It is believed that deconjugation takes place in the terminal ileum, and both deconjugation and dehydroxylation in the large intestine (Matern & Gerok, 1979).

The toxic effects of lithocholic acid include alteration of liver structure in a number of mammalian species following oral administration of the acid itself or the glycine or taurine conjugates of lithocholic acid. Intra-muscular injection of lithocholic acid in man will produce pyrogenic reactions (Percy-Robb, 1975). The lysis of human erythrocytes *in vivo* has been demonstrated (Palmer, 1964).

In man, the major bile acids are cholic, chenodeoxycholic and deoxycholic with very little of the other secondary bile acid, lithocholic, owing to its insolubility even when conjugated. In the rat, the principal bile acid is cholic acid, with chenodeoxycholic and ursodeoxycholic (3α,7β-dihydroxy-5β-cholanic) acid also occurring.

Bile acid or bile salt

These terms are commonly used interchangeably. Although the state of ionisation of the molecule is the strict criterion, such that a bile acid is unionised and a bile salt ionised, further confusion arises from reference in the literature to free, unconjugated molecules as bile acids, and bile salts as conjugated molecules. It is the latter definition which will be used on most occasions in this thesis. It should be remembered that at physiological pH, most conjugated and unconjugated bile acids exist as sodium salts.

BILE ACID-BINDING PROTEINS

The enterohepatic circulation of bile acids

The enterohepatic circulation (Figure 1.2) is responsible for the recycling of bile acids between the liver and intestine. In man, after synthesis and conjugation in the liver, bile salts are secreted into bile and pass down the bile duct to the gall bladder, where the bile is concentrated by a factor of 5-10 with respect to organic components (Davenport, 1966). This concentration is achieved by removal of water and electrolytes, mainly by a coupled active absorption of sodium and chloride ions (Dietschy, 1966). In a cholecystokinin-mediated response to eating, the gallbladder contracts and discharges its contents into the third part of the duodenum (Meyer & Grossman, 1972). The rat does not possess a gall bladder.

Intestinal events

In the small intestine, bile salts aid the digestion and subsequent absorption of fat and its digestive products (see p.4-5). Some of the bile salts will be deconjugated and/or dehydroxylated by bacterial action; together bile salts and bile acids are reabsorbed from the intestine, mainly at the terminal ileum, and also from the colon. This process involves both active and passive mechanisms.

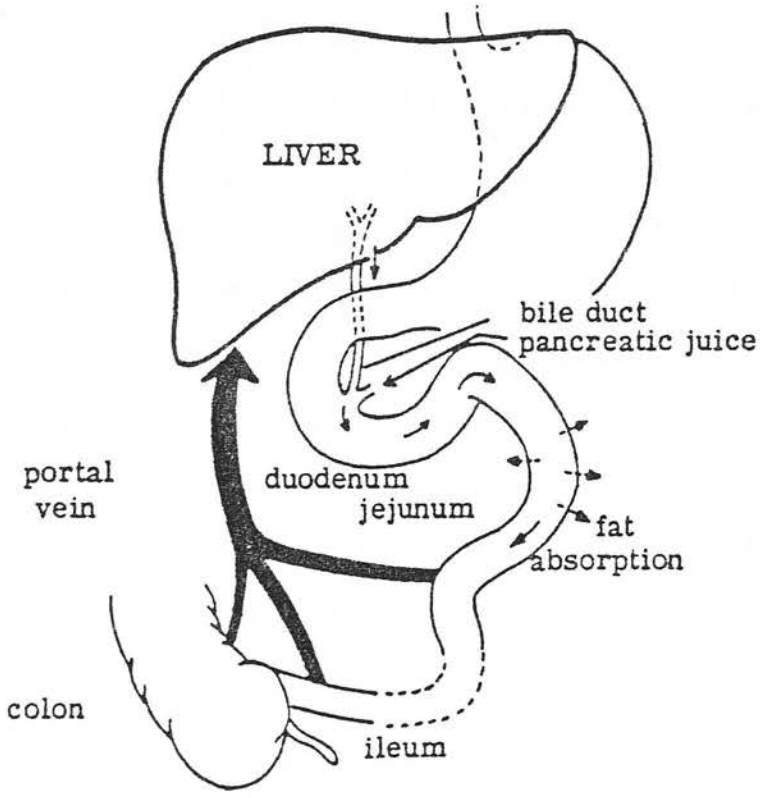


FIGURE 1.2 The enterohepatic circulation

Active intestinal transport

Some 70-85 per cent of bile acids are reabsorbed at the terminal ileum (Tyor, 1971; Hepner et al., 1972b). The mechanism involved is concentrative and can be blocked by metabolic inhibitors and hypoxia (Lack & Weiner, 1961; Holt, 1964; Glasser, 1965; Gallagher et al., 1976). In addition, the uptake exhibits saturation, is sodium-dependent and competition between bile acids can be demonstrated (Holt, 1964; Schiff et al., 1972; Krag & Phillips, 1974; Gallagher et al., 1976). It is believed that the active site of the ileal transport system contains a positive charge for electrostatic interaction with anionic bile salts and a negative charge associated with sodium ions (Gallagher et al., 1976; Bundy et al., 1977). The arrangement of these charges is such that both the bile salt and sodium ion occupy the membrane transport site simultaneously in a co-operative manner.

Photoaffinity labelling experiments (see p.77) with brush-border membrane vesicles from the jejunum and ileum of the rat have revealed several bile salt-binding polypeptides, which are listed in Table 1.1 (Kramer et al., 1983). These polypeptides, speculated to be involved in the transport of bile salts out of the intestine, are distinct from those found in the hepatocyte using the same technique (see Table 1.2).

Area of small intestine	Molecular masses of bile salt-binding polypeptides (Da)
Ileum	125 000; 99 000; 83 000; 67 000; 43 000
Jejunum	125 000; 94 000; 83 000; 67 000; 43 000

Table 1.1 Bile salt-binding polypeptides from rat small intestine revealed by photoaffinity labelling.

Together, these results are consistent with the existence, in the terminal ileal brush-border mucosa of the rat, of a carrier-mediated, energy-linked sodium-bile acid co-transport system.

Passive intestinal absorption

Passive absorption may occur at any point in the small or large intestine, relying on ionic or non-ionic diffusion (Dietschy et al., 1966; Schiff, 1972; Wilson & Dietschy, 1972). Intraluminal pH, the dissociation constants (pKa) of individual bile acids, and lipid solubilities of ionic and non-ionic species are all factors that determine which of the two diffusion processes prevail. At physiological pH (5.0-7.0), more unconjugated bile acids will be non-ionized (pKa 5.5-6.5) than glycine-conjugated bile acids (pKa 3.8-4.8), whereas taurine-conjugated bile acids (pKa 1.85-1.95) will be almost entirely in the ionized form (Small, 1971). Thus, free bile acids are absorbed more rapidly from extra-ileal sites than glycine conjugates, and taurine conjugates are almost totally dependent on the active transport system in the ileum (Hislop et al., 1967; Dowling et al., 1972). Almost total reabsorption of bile acids from the intestine is achieved by the active and passive transport systems together. However, small amounts of bile acids are not reabsorbed, being finally excreted in the faeces after bacterial transformation and yielding as many as 30 dif-

ferent bile acids (Eneroth et al., 1966a,b). Bile acids, particularly dihydroxy forms, inhibit water reabsorption by the colon, and thus under conditions of bile acid malabsorption, severe diarrhoea can occur (Hofmann & Poley, 1972).

Hepatic events

Bile acids and salts are returned to the liver, after absorption, in the portal blood bound mainly to albumin (Rudman & Kendal, 1957; Kakis et al., 1979). They are extracted from the blood by hepatic parenchymal cells and transported across the hepatocyte. During their passage back through the liver, free bile acids are reconjugated very efficiently, both cholic acid and chenodeoxycholic acid being completely conjugated in a single pass (von Bergmann et al., 1975). Mice and rats also possess a large capacity for the rehydroxylation of conjugated deoxycholic acid at the 7α-position, the reaction being catalyzed by the microsomal fraction and requiring NADPH, molecular oxygen and cytochrome P-450 (Prager et al., 1973). Human liver, in contrast, appears unable to rehydroxylate reabsorbed bile acids to any great degree (Einarsson & Hellstrom, 1974).

After crossing the liver, the bile salts are resecreted against a concentration gradient into the bile canaliculi (O'Maille et al., 1965, 1967). In this manner, the enterohepatic circulation conserves the body's

pool of bile acids and bile salts, estimated to be between 2g and 5 g, which circulates between liver and intestine two to three times per meal (Borgstrom et al., 1957; Small et al., 1972). The bile acid pool is kept in a steady state. In man, the liver synthesises approximately 500-800 mg of bile acids per day; faecal losses amount to 200-800 mg per day (Borgstrom et al., 1962; Grundy et al., 1965). In the rat, the bile acid pool is 20 mg, and about 200 mg of bile salt and bile acid are transported daily across the liver (Cronholm & Sjovall, 1967).

Hepatic bile acid transport

The transport of bile acids across the liver can be divided into three phases as follows - transport across the hepatocyte sinusoidal membrane, transport across the hepatocyte itself and transport across the canalicular membrane. This thesis is primarily concerned with the intracellular transport of bile acids.

1) Uptake across sinusoidal membrane and secretion across canalicular membrane

Owing to technical difficulties with hepatic membrane preparations, it has so far proved impossible for workers to obtain "pure" preparation of sinusoidal or canalicular membranes. Preparations enriched in one or the other of these two membranes is the best that has so far been achieved. As a result of this, and owing to apparent similarities between the two processes, transport of bile acids across the sinusoidal and canalicular membranes will be covered together, in one section.

The arrangement of hepatocyte parenchymal cells along the sinusoidal lumen is shown in Figure 1.3. The fenestrations of the lining cells allow free access for even large molecules to the space of Disse and facilitate uptake of substances, such as bile acids, which circulate in plasma tightly bound to albumin. The sinusoidal transport system has been proposed to be carrier-mediated following various studies in the dog, in isolated perfused rat liver and in isolated rat liver cells (Glasinovic et al., 1975; Reichen & Paumgartner, 1975, 1976; Schwarz et al., 1975; Reichen et al., 1977). In these cases, the bile acid transport system has exhibited Michaelis-Menten kinetics, bile acid specificity, competition between the bile acids and sodium dependence.

Although the molecular components of this transport system are not known, bile acid receptors in isolated rat liver sinusoidal (and canalicular) membranes have been reported (Accantino & Simon, 1976). Also, a high affinity site for cholic acid has been described in isolated rat liver plasma membranes (Anwer et al., 1977). Competitive inhibition has been demonstrated between different bile acids, suggesting a common pathway which is distinct from that by which organic anions enter the hepatocyte (Paumgartner & Reichen, 1975; Ohkuma & Kuriyama, 1982).

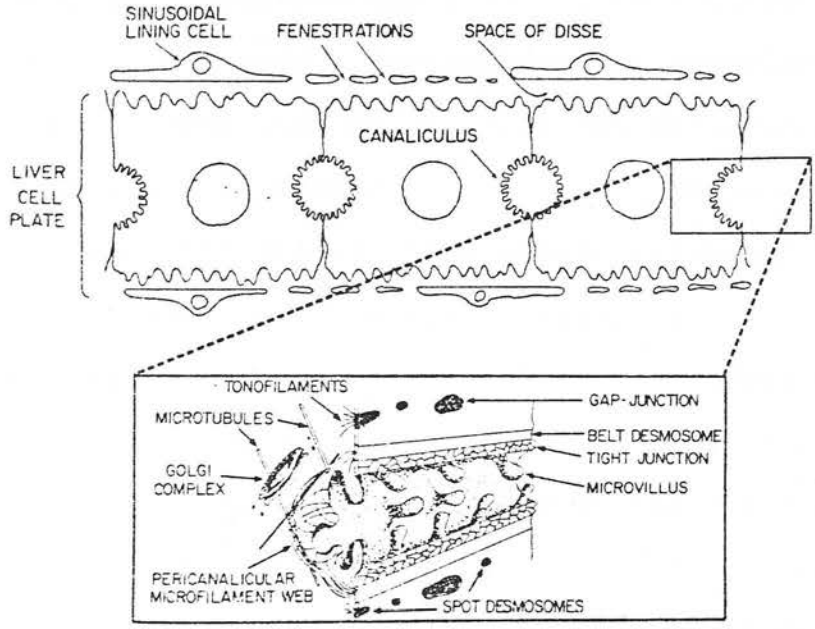


FIGURE 1.3 Liver cell plate

Schematic illustration of the arrangement of hepatocytes in single cell-thick sheets within the liver.

Anwer and Hegner (1978a) suggest that bile acid uptake consists of three components - (a) non-saturable, Na^+-independent (probably diffusion); (b) Na^+-independent, saturable and (c) Na^+-dependent saturable - and that as a result of inhibition and competition studies, cholate and taurocholate share a Na^+-independent transport system, while taurocholate has at least two Na^+-dependent systems, one of which is shared by cholate. These authors also speculate that the Na^+-independent uptake of bile acids is a common carrier for organic anions, e.g. bromosulphthalein and bilirubin, whilst the Na^+-dependent system is specific for bile acids (Anwer & Hegner, 1978b).

The uptake capacity (Vmax) of the rat liver for bile acids greatly exceeds the excretory transport maximum (Tm) in the steady state (Glasinovic et al., 1975; Paumgartner, 1975; Reichen & Paumgartner, 1975, 1976; Poupon et al., 1976). This step, then, is not rate-limiting in bile acid transport from blood to bile. Recent studies have provided evidence for a lobular gradient of bile acid uptake by hepatocytes, transport across the sinusoidal membrane being greater at the periportal than the pericentral region of the liver lobule (Jones et al., 1980b).

Transport of bile acids into the canaliculus is also believed to be carrier-mediated as it is saturable and a transport maximum has been measured (Paumgartner et al., 1974, 1975; Poupon et al., 1976; Schwarz et al., 1976).

This step is rate-limiting in the overall transport of bile acids from blood to bile (Paumgartner et al., 1975; Poupon et al., 1976).

Experiments with photoaffinity probes (see p.39) and hepatocyte plasma membrane fractions enriched either with sinusoidal surfaces or canalicular membranes, have demonstrated the presence of bile salt-binding polypeptides in these membranes. These are listed in Table 1.2 (Kramer et al., 1980; Abberger et al., 1981; Fricker et al., 1982; Kramer et al., 1982). Of these, the 20 000 Da polypeptide contributes little to the total bile salt-binding capacity and the 67 000 Da polypeptide has been shown to be albumin. Other workers have used the same technique with both intact hepatocytes and membrane preparations enriched with sinusoidal surfaces to reveal a polypeptide of approximate molecular mass 54 000 Da (Wolkoff & Chung, 1980; von Dippe et al., 1983; von Dippe & Levy, 1983), which is believed to be a component of the rat hepatocyte bile acid transport system. Photoaffinity labelling of intact hepatocytes from tissue cultures of hepatoma cells known to lack bile acid transport activity have failed to reveal this protein (von Dippe & Levy, 1983). A bile salt-binding polypeptide of molecular mass 43 000 has also been demonstrated in intact rat hepatocyte plasma membranes by other workers (Cheng & Levy, 1979).

Membrane	Molecular masses of bile salt-binding polypeptides (Da)
Sinusoidal)))) Canalicular)	67 000; 54 000; 48 000; 43 000; 20 000

Table 1.2 Bile salt-binding polypeptides from rat hepatocyte plasma membranes (sinusoidal and canalicular) revealed by photoaffinity labelling.

A working model of canalicular bile formation has been proposed and is shown in Figure 1.4 (Boyer, 1980). The model proposes that a Na+-K+-adenosine triphosphatase (ATPase) on the sinusoidal and lateral hepatocyte membranes extrudes sodium from the cell, thereby creating an electrochemical gradient. A carrier in the sinusoidal membrane couples the transport of sodium and bile acids into the cell, thus concentrating bile acids within the hepatocyte. Subsequent entry of bile acids into the canaliculus, whether carrier-mediated or as a result of vesicular transport (see p.29) is against a concentration gradient and is thus energy-requiring (Erlinger et al., 1970). Sodium, and also other inorganic electrolytes, organic solutes and water, may enter the canaliculus by crossing the tight junctions.

2) Intra-cellular transport of bile acids

The pathway which bile acids take once inside the hepatocyte, i.e. from sinusoidal to canalicular membrane, is unknown. There are several alternatives (a) bile acids might cross the cell partitioned between organelle membranes and the cytosol; (b) vesicular transport; (c) association with cytosolic carrier proteins (Strange et al., 1979a,b,c).

(a) Partitioning

By studying the different degrees to which various bile acids partition into subcellular organelles, or are

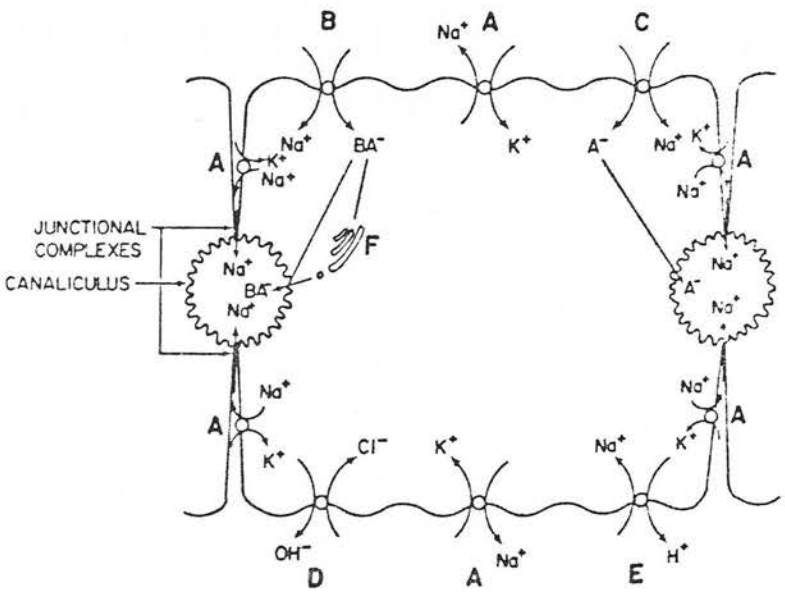


FIGURE 1.4 Model of canalicular bile formation

BA = bile acids; A = Na^+-K^+-ATPase;

B,C = anion-sodium coupled transport systems;

D = hydroxyl-anion, E = protein-sodium ion exchangers; F = vesicular transport.

bound to cytosolic protein(s), it has been possible to calculate a diffusion coefficient, D (Smallwood et al., 1974; Strange et al., 1979a,b,c). The magnitude of D, when compared to observed transit times for bile acids crossing the liver, is compatible with bile acids traversing the hepatocyte in free solution, the rate of passage being proportional to the concentration of bile acid in free solution (Strange et al., 1979b). This, however, is a mathematical concept which has not been proven.

(b) Vesicular transport

Recent studies have shown that the protein components of bile may cross the hepatocyte in membrane-bound vesicles (Renston et al., 1980a,b). This concept has been extended to bile acids themselves, supported by the observed increase in pericanalicular vesicles during bile salt-induced choleresis (Boyer et al., 1979; Jones et al., 1979). The Golgi complex has also been implicated in the intra-hepatic transport of bile acids (Jones et al., 1976a,b; Jones & Spring-Mills, 1977). A model linking these ideas has been proposed in which the bile acids are taken to the endoplasmic reticulum and Golgi, possibly by a carrier protein, and after processing are taken to the canaliculus in vesicles and excreted into bile (Goldmsith et al., 1983). The Golgi complex and bile secreting process have previously been linked (Jones et al., 1980a).

(c) Cytosolic binding proteins

A large number of compounds, including organic anions such as bilirubin, bromosulphthalein and indocyanine green, as well as bile acids, are transported across the hepatocyte, and may be bound to cytosolic protein. Gel filtration of rat hepatic cytosol mixed with bilirubin results in three peaks of binding - X (eluted in the void volume, - Y (Mr approximately 45 000 Da) and Z (Mr approximately 12 000 Da) - the majority of the binding being associated with the Y and Z peaks (Levi et al., 1969). Bile salt binding to the latter has been recognised for a number of years - Z protein is also known under several other names (Ockner et al., 1972; Ketterer et al., 1976; Dempsey et al., 1981). Table 1.3 lists the organic anion binding polypeptides found in rat hepatic cytosol.

The Y peak was found to contain a group of detoxification enzymes, the glutathione S-transferases (EC.2.5.1.18), which collectively represent 3-8% of the total cytosolic protein (Kaplowitz et al., 1973; Habig et al., 1974; Jakoby et al., 1978). These enzymes catalyse the conjugation of glutathione with a large number of electrophiles, and also bind hydrophobic compounds that are not substrates, such as bile acids (Strange et al., 1977; Hayes et al., 1979, 1980; Pattinson, 1981; Vessey & Zakim, 1981). The rat hepatic transferases, which are

dimeric proteins, each comprise two of five subunits - Ya (Mr 25 500), Yn (26 500), Yb_1 and Yb_2 (27 000) and Yc (28 500) (Bass et al., 1977; Hayes et al., 1980; Scully & Mantle. 1981; Hayes, 1984; Pickett et al., 1984). These enzymes have been shown to be inhibited to differing extents by bile salts, namely cholate, chenodeoxycholate and lithocholate-3-sulphate (Hayes & Chalmers, 1983). The physiological significance of bile salt-binding by the glutathione S-transferases is not clear; they may participate in the intrahepatic transport of both newly synthesized and recycled bile acids or they may act to restrict the amount of bile acid available for partitioning into subcellular organelles and protect the hepatocyte from the potentially damaging effects of bile acids (Strange, 1981). Alternatively, they may minimise the back diffusion of bile acids in the hepatocyte (Wolkoff et al., 1979).

High resolution gel filtration of rat hepatic cytosol incubated with radioactive bile acids has revealed a further bile salt-binding fraction within the Y peak, which has been termed Y' (see Table 1.3) containing polypeptides in the molecular mass range 30 000 - 40 000 Da (Sugiyama et al., 1982, 1983). Further purification has apparently resolved a total of six binding proteins, four of which bind organic anions, Dv, D_1, D_2 and D_3, and also two distinct bile acid-binding proteins, I and II. These

Fraction	Alternative Names	Molecular Mass (Da)
X	No further study; possibly membrane fragment	> 100 000
Y	Ligandin; Glutathione S-transferase	45 000 - 56 000
Y'	D_Y, D_1, D_2 and D_3; Bile acid binders I and II	30 000 - 40 000
Z	Fatty acid-binding protein; Amino azo dye-binding protein A; squalene and sterol carrier protein	12 000 - 14 000

Table 1.3 Organic anion-binding polypeptides in rat hepatic cytosol.

latter proteins are monomeric, with a molecular mass of approximately 33 000 Da and isoelectric points of 5.6 and 5.5 respectively. Although they have very similar amino acid compositions, the genetic relationship between these two bile acid-binding proteins is unknown. The two binders have been shown to possess different binding characteristics, II generally having a higher affinity for conjugated bile acids as measured by amino-naphthalene sulphonate (ANS) fluorescence inhibition (Sugiyama et al., 1983).

Experiments with isolated hepatocytes and bile acid-derivatives used as photoaffinity probes have shown no interaction between glutathione S-transferases and bile acids under physiological conditions (Abberger et al., 1983). However, using rat hepatic cytosol, the same technique has labelled, with conjugated or unconjugated derivatives, five or six polypeptides, as listed in Table 1.4 (Abberger et al., 1981). Of these polypeptides, the 67 000 Da polypeptide is thought to be albumin, the 23 000 Da polypeptide a glutathione S-transferase subunit, and the 43 000 Da polypeptide has been identified as a subunit of hydroxycholanoyl transferase (EC.2.3.1.?) an enzyme which catalyses the final step in the synthesis of conjugated bile salts (Abberger et al., 1981). The remaining proteins have still to be identified.

Bile acid-derivative used for labelling	Molecular masses of bile salt-binding polypeptides (Da)
Conjugated	67 000; 43 000; 39 000; 32 000. 23 000
Unconjugated	67 000; 43 000; 39 000; 32 000; 23 000; 15 000

Table 1.4 Bile salt-binding polypeptides from rat hepatic cytosol revealed by photoaffinity labelling.

INVESTIGATION OF BILE ACID-BINDING

There are a variety of techniques available for the study of ligand-binding to proteins generally which can be applied specifically to bile acid-protein binding. These include (a) equilibrium dialysis; (b) equilibrium gel filtration chromatography; (c) affinity chromatography; (d) enzyme inhibition; (e) photoaffinity labelling. The last of these methods has played an important role in this project.

Equilibrium dialysis

This method involves the use of a semi-permeable membrane to separate two solutions, one of which contains the putative binding protein, the other a ligand which may bind to the protein. Provided the ligand involved is small enough to cross the semi-permeable membrane during dialysis, and that an assay exists to measure the ligand concentration, this technique allows the investigation of ligand-protein binding and the calculation of a dissociation constant, K, for the ligand to the protein. Both solutions, separated by the semi-permeable membrane, are arranged within a dialysis cell, which is rotated in a water bath at, for example, 37°C for several hours until equilibrium within the system has been achieved. At this point, the concentration of free ligand on both sides of the membrane will be equal. However, if the protein binds the ligand, then the total concentration of ligand

on the protein side will be greater. Thus, the dissociation constant for ligand can be expressed in measurable quantities as follows:

$$K = \frac{[\text{free ligand}][\text{protein}]}{[\text{total ligand} - \text{free ligand}]} \quad *$$

This method has been used to investigate bile acid-binding to proteins (Strange et al., 1976; 1979a). However, the sensitivity is not great and relatively large amounts of protein are required.

Equilibrium gel filtration chromatography

The use of this technique to investigate protein-ligand binding was first described by Hummel & Dreyer (1962). A gel filtration column (commonly Sephadex G-25, chosen so as the protein is excluded from the matrix) is equilibrated with a buffer containing a ligand which will bind to the protein. The protein under test is dissolved in the same buffer, containing the ligand, and applied to the column. As the protein moves down the column, it will bind the ligand. As the zone of protein and protein-ligand complex continues down the column, it will move into a fresh solution of ligand, forming more of the complex. This process continues until an equilibrium is reached, at which point the concentration of free ligand in the protein-ligand complex zone is the same as in the buffer. However, as the protein has bound the ligand, the total concentration of ligand in the zone is higher than

*NOTE: This equation applies only to a molecule with a single binding site. A more complex equation is required for those molecules with multiple sites.

its initial concentration in the buffer. A corresponding zone of decreased concentration of ligand moves down the column at the rate of which the free ligand moves; the extent of ligand binding to the protein can be assessed by the excess in the total ligand concentration in the protein-ligand complex zone or the deficit of ligand in the zone of decreased concentration of ligand.

This technique has been used to study bile acid-binding proteins, although problems exist in obtaining a stable baseline (Hayes et al., 1980).

Affinity chromatography

Affinity chromatography utilizes the inherent biological specificity of a ligand-protein interaction to effect the separation of ligand-binding proteins from a complex mixture. A ligand is immobilised on an insoluble support, such as the gel matrix Sepharose, by covalent attachment. The gel, coupled with ligand, is then packed into a column. Once the mixture of proteins has been passed down the column, those which are bound via protein-ligand interaction are eluted by passing a solution of free ligand down the column. The free ligand competes for binding sites on the protein with the immobilised ligand, and the protein(s) is thus eluted from the column.

Rat hepatic glutathione S-transferases have been investigated on an affinity column where cholic acid has been covalently attached via a carbodiimide reaction

(Pattinson et al., 1980; Pattinson, 1981a,b,c). Another immobilised ligand which has been employed is glutathione, in the study of glutathione S-transferases from both rat and human liver and human placenta (Simons & Vander Jagt 1977; Vander Jagt et al., 1981, 1982).

Enzyme inhibition

With purified bile acid-binding proteins such as the glutathione S-transferases, which also possess enzyme activity, it is possible to study the extent to which different types and different concentrations of bile acids will inhibit the enzyme activity. This may yield information, for example, regarding the relative affinity of the binding protein for various bile acids, and also the type of inhibition involved in each case (Vessey & Zakim, 1981; Hayes & Chalmers, 1983). A disadvantage with this method is that the substrate-binding site and ligand-binding site of the enzyme must either be one site, or if not, be close enough together for ligand-binding to perturb enzyme activity.

Binding proteins do not, however, always possess enzyme activity; in such cases the technique of inhibition of fluorescence, either intrinsic or extrinsic, may be applicable. For proteins, there are only three intrinsic fluors - tryptophan, tyrosine and phenylalanine, in order of decreasing strength of fluorescence. If, for example, it is known that one of these amino acids is present at

the ligand-binding site of the protein, then the change in fluorescence of that particular fluor which occurs on the addition of ligand to the protein solution, may provide data on the structure of the ligand-binding site and events which occur there.

Not all binding proteins under investigation possess a conveniently placed fluorescent group. However, it is possible to introduce an extrinsic fluor into the binding protein, such as 1-anilino-8-naphthalene sulphonate (ANS), which will bind to the ligand-binding site of the protein. The inhibition of fluorescence of the ANS by the ligand can then be studied in the same manner as with intrinsic fluors. This has been done with marine elasmobranch hepatic Z-protein and also with the newly discovered class of rat hepatic bile acid- and organic anion-binding proteins, Y′ (see p.31) (Sugiyama et al., 1982, 1983).

Photoaffinity labelling

Affinity probes offer another approach in the study of ligand-binding sites of proteins. Using this technique, the active site of the binding species is covalently linked to the ligand. The principle of the method is the alteration of the ligand so as to incorporate a chemically labile group, which will then irreversibly react with the protein after binding, without disturbing the ligand-receptor recognition process. The ligand then becomes an

affinity label, theoretically able to selectively modify the binding site of the protein via the inserted chemically labile group (Bayley & Knowles, 1977). This approach, however, has two limitations. The first is that the chemical reactivity of the ligand must be designed so as not to react rapidly with water and thus be destroyed by hydrolysis. Secondly, such affinity labels do not permit the investigation of the internal components of a cell, vesicle or organelle, as they will react initially with external components.

The solution to these problems lies in being able to activate the label once it is in the desired position, and this has been achieved through the use of photoactivatable reagents. Two types of such reagents have so far been employed. The first involves covalent attachment of the photoactivatable group to the protein, followed by irradiation with light (photolysis) to activate the group. This approach was developed in the first studies performed with photoactivatable ligands, using a diazoacetyl derivative of chymotrypsin (Singh *et al.*, 1962).

The second group of photoactivatable reagents used, which are more generally applicable, have been termed photoaffinity labels (Kiefer *et al.*, 1970). This method employs a reversibly bound ligand which has been chemically altered to introduce a group capable of photoactivation. Incubation of such a molecule with a putative

binding protein, followed by photolysis, will generate a highly reactive intermediate that covalently attaches to the binding site of the protein. The ligand can be radiolabelled, thus allowing subsequent analysis of the binding, and investigation of the biochemical structure of the binding site. As this technique is based on protein-ligand affinity, its specificity will be high (Bayley & Knowles, 1977).

Over the last two decades, photoaffinity labelling has become increasingly valuable as a technique for the investigation of biochemical systems at the molecular level. Such work has included the study of receptors and transport proteins, membrane structure and function, protein- and drug-nucleic acid interactions, antibodies and enzymes (Chowdhry et al., 1976; Cantrell & Yielding, 1977; Guillory et al., 1977; Jakoby & Wilchek, 1977; Rosenblit et al., 1977; Bisson et al., 1978). There have also been several comprehensive reviews of the photoaffinity labelling of proteins (Jori, 1973; Creed, 1974; Cooperman, 1978; Glazer et al., 1976).

Types of photoactivatable groups

A photoactivatable group is that part of the chemically-stable precursor of a photoaffinity label, which, on photolysis, forms a highly reactive intermediate. This can occur by homolytic cleavage, either at a single bond to yield two free radicals or a diradical,

or at a double bond to carbon and nitrogen, resulting in the formation of a carbene or nitrene respectively (Bayley & Knowles, 1977). The chemistry of the former mechanism is currently ill-defined. The most easily synthesized and best understood intermediates are nitrenes and carbenes, the photogenerated products of diazo compounds and azides respectively. These highly reactive intermediates can attack a variety of amino acid side-chains, including the hydrocarbon type.

Azides (R-N=N-=N+) and diazo compounds (R-C=N-=N+) are organic nitrogen compounds with a system of cumulative multiple bonds. They both possess weak absorption bands at wavelengths above 400 nm, and stronger absorption bands at approximately 270 nm, tailing off toward 350 nm. Figure 1.5a,b shows the photo-elimination of nitrogen due to homolytic bond cleavage, which occurs after visible or near ultraviolet irradiation of such compounds.

Carbenes

Carbenes react very rapidly in several different ways and have been widely used (Singh *et al.*, 1962; Shafer *et al.*, 1966; Vaughn & Westheimer, 1969; Browne *et al.*, 1971; Hexter & Westheimer, 1971; Jones & Moss, 1973; 1975). They may attack nucleophilic centres, add to multiple bonds, insert into single bonds, and by hydrogen abstraction, form free radicals which may then couple (Figure 1.5b) (Bayley & Knowles, 1977).

a) $R_1-N=N=N + hv \longrightarrow R_1-\ddot{N}: + N_2$

b) $R_2-CH=N=N + hv \longrightarrow R_2-\ddot{C}H + N_2$

c) $R-CH_2-\ddot{C}H \longrightarrow R-CH=CH_2$

d) $R-\underset{\underset{O}{\|}}{C}-\ddot{C}H \longrightarrow R-CH=C=O$

e) $R'-\underset{\underset{O}{\|}}{C}-CR=\overset{+}{N}=\overset{-}{N}$ 1

$R-\underset{\underset{O}{\|}}{C}-CH=\overset{+}{N}=\overset{-}{N}$ 2

$R-\underset{\underset{O}{\|}}{C}-\overset{COOR'}{\overset{|}{C}}=\overset{+}{N}=\overset{-}{N}$ 3

$Ar-HC\begin{matrix}N\\ \|\\ N\end{matrix}$ 4

FIGURE 1.5 Photoactivatable groups

(a) Azide to nitrene;

(b) Diazo compound to carbene;

(c) Hydrogen migration in carbene;

(d) Wolff rearrangement in carbene;

(e) Carbene precursors.

Potential problems with the use of carbenes as photoaffinity probes include the presence of a hydrogen atom on the carbon atom adjacent to the carbon centre (Figure 1.5c). Hydrogen migration may occur, leading to an unreactive olefin. Also, the intramolecular Wolff rearrangement may occur - the example given is an α--ketocarbene, yielding an unreactive ketone (Figure 1.5d). Several chemically-stable, easily-synthesized precursor species which form carbenes on photolysis are shown in Figure 1.5e. These include - α-diazoketones, α-diazoacetyl- and α-diazomalonyl-compounds, and aryldiazimines (Hexter & Westheimer, 1971; Hixson & Hixson, 1972; Jones & Moss, 1973, 1975; Smith & Knowles, 1975a,b). In the latter, the degree of intramolecular rearrangement is substantially lower than in other diazo compounds (Bayley & Knowles, 1977).

Nitrenes

Nitrenes are the nitrogen analogues of carbenes and were first used in the photoaffinity labelling of antibodies (Fleet et al., 1969). The range of reactions that nitrenes may undergo is broadly similar to that of carbenes, although they are more selective in the reactions and their reactivity is considerably lower than carbenes (Bayley & Knowles, 1977). Figure 1.6a illustrates the types of reactions nitrenes will participate in - insertions into C-H bonds to give secondary amines, cycload-

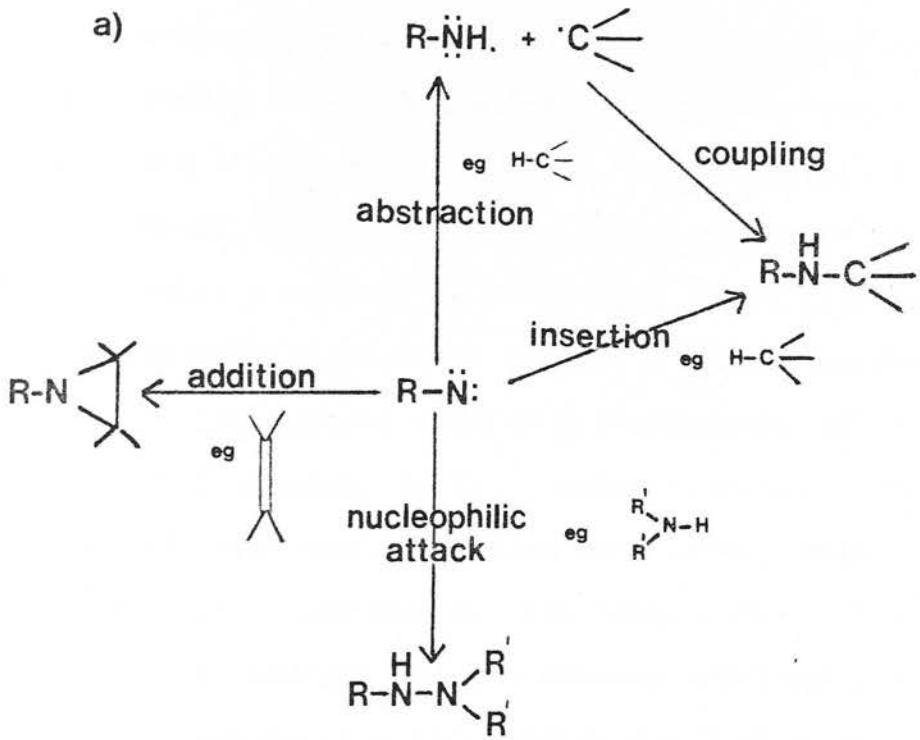
ditions to double bonds to form cyclic 3-member imines, addition to nucleophiles and hydrogen atom abstraction followed by coupling of the free radicals. Nitrenes also tend to be more electrophilic and prefer an O-H to a C-H bond.

Several types of precursors - acyl, alkyl and aryl azides - yield nitrenes upon photolysis (Bayley & Knowles, 1977). However, by far the most frequently used nitrene source is an aryl azide (Figure 1.6b). Acyl azides are powerful acylating agents and the photogenerated nitrenes tend to rearrange. Alkyl azides are chemically unstable and short-wavelength ultraviolet light, which may be biologically damaging, is needed for photolysis. Aryl azides, however, are stable in the dark, can be photolysed at longer wavelengths and the resulting nitrenes are not susceptible to intramolecular rearrangement. These factors, together with their greater stability to changes of pH and temperature, make aryl azides an attractive alternative to diazo compounds for photoaffinity labelling (Fleet et al., 1969; Knowles, 1972).

Photolysis

Irradiation of the photoaffinity label presents two problems - (a) production of ultraviolet, near ultraviolet or visible light, and (b) selection of appropriate wavelength for smooth, fast, efficient photolysis.

a)



b)

alkyl azides eg methyl azide
n-butyl azide

acyl azides eg acetyl azide
ethyl azidoformate

aryl azides eg p.methoxyphenyl azide
2-azido-3-nitro biphenyl

FIGURE 1.6 Nitrenes

(a) Reactions of nitrenes;

(b) Nitrene precursors.

Several different irradiation procedures have been reported. Photolysis of diazocetyl chymotrypsin was achieved using three sunlamps, with all radiation below 315 nm filtered out with a solution of copper sulphate pentahydrate (Singh et al., 1962). Fleet et al. (1969) used two Mazda 125 W pearl glass lamps for their studies. More recently, a variety of commercial photolysis reactors have become available, which generally employ mercury arc lamps as a light source (Hexter & Westheimer, 1971; Cooperman & Brunswick, 1973). Other sources, such as cadmium arc, zinc arc and helium neon laser, emit at one or more specific wavelengths (see Table 1.5). The most commonly used sources are the mercury arc lamps, of which there are three types - low, medium and high pressure.

The low pressure mercury arc operates at low power (approximately 25 watts) and emits most of its light at 254 nm - these lamps are commonly known as germicidal. The medium pressure mercury arc requires more power (100 -500 watts), operates at a higher temperature, and therefore needs to be cooled. In this lamp, the 254 nm line is suppressed, although several other characteristic lines, especially 313 nm and 316 nm, are emitted. Generally, these lines are broad, and superimposed on a background emission. Figure 1.7 shows spectral outputs of a low and medium pressure mercury arc lamp. High pressure mercury arcs work at power up to several kilowatts, giving strong output at most wavelengths.

SOURCE	EFFECTIVE WAVELENGTH RANGE (nm)
(a) Weak	
Tungsten lamp	450 to visible
Hydrogen lamp	165 to visible
Carbon arc	400 to visible
(b) Intermediate	
Mercury arc (low pressure)	185, 254
Cadmium arc	229, 326
Zinc arc	214, 308
(c) Strong	
Sunlight	340 to visible
Mercury arc (med. pressure)	200 to visible
Mercury arc (high pressure)	240 to visible
Xenon arc	200 to visible
Helium neon continuous laser	633

Table 1.5 Light sources for photolysis.

The choice of wavelength suitable for photolysis of a particular label is obviously restricted by the absorption spectrum of the molecule. For example, diazoketone and diazoacetyl derivatives have two absorption bands - at 254 nm (ε 7000) and 350 nm (ε 10). The intermediate formed at 254 nm is more reactive than the one generated at the longer wavelength, thus increasing the chance of covalent attachment to the protein. However, proteins are subject to photodecomposition at lower wavelengths; as a result, irradiation at 350 nm would probably be more suitable. Proteins with a low cystine or tryptophan content, however, are apparently not extremely sensitive to 254 nm radiation (Cooperman & Brunswick, 1973). Thus, each particular protein should be studied for sensitivity to photodecomposition prior to photoaffinity labelling.

To select a particular wavelength or range of wavelengths, filters can be employed. These can take the form of either chemical solutions or glass window materials. The former tend to isolate single emission lines, whereas the latter tend to have "cut-offs", blocking out radiation below a particular wavelength (Nielsen et al., 1983) (Table 1.6).

The photolysis system used in this study is an annular photoreactor, shown in schematic form in Figure 2.3, and described more fully in the Methods section (p. 75).

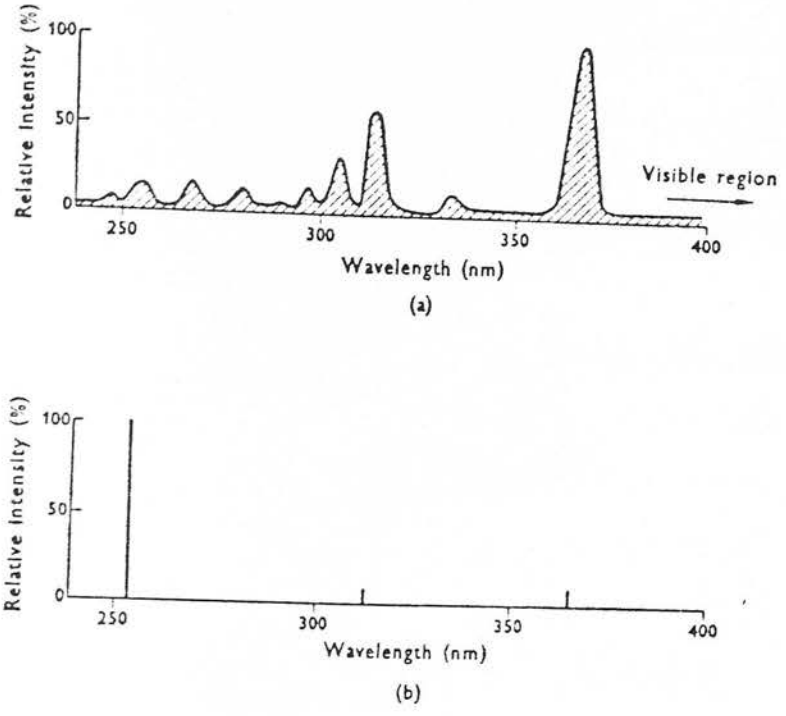


FIGURE 1.7 Spectral outputs of (a) medium pressure and (b) low pressure mercury arc lamps.

Photoaffinity label design

There are several criteria that a molecule should fulfil in order to be successful as a photoaffinity label:-

(1) the precursor should be easily synthesized and chemically stable;

(2) such a molecule should photolyse smoothly at wavelengths which are not damaging to other components of the reaction system;

(3) the intermediate species generated by photolysis should be highly reactive, have a very short half-life and not be liable to intramolecular rearrangement to a less reactive compound (Bayley & Knowles, 1977; Jori & Spikes 1977).

Consideration of structural requirements for ligand-binding should precede design and synthesis of the photoaffinity label (Bayley & Knowles, 1977). More successful labelling will be achieved if the binding of the photolabile ligand to the protein is tight. Obviously if the lifetime of the photogenerated intermediate is long and its exchange rate between protein and solution high, then the extent and specificity of labelling will be affected. In such cases, photolabelling may be performed in the presence of scavengers, which are materials intended to destroy all photogenerated intermediates other than those at the ligand-binding site. Some examples of scavengers are p-aminobenzoic acid, hydro-

Wavelengths (nm)	Thickness	Solution/Window Material
254	+3 cm	Chlorine gas at 100 kPa pressure
	+1 cm	Iodine-potassium iodide 0.01% I_2, 0.014% kI
	5 cm	Nickel and cobalt sulphate 35%, 1% respectively
313	+1 cm	Potassium hydrogen phthalate 0.5%
	4 cm	Nickel and cobalt sulphate 35%, 10% respectively
366	5 cm	Copper sulphate, 12.5%
	+2 mm	Chance-Pilkington OX-7 or Kodak Wratten 18B
>450 nm		Corning 3-72 filter
>370 nm		WG-375 filter
>330 nm		Acetone
>300 nm		Pyrex filter

Table 1.6 Filter solutions and window materials for photolysis.

quinone, soluble proteins, p-aminophenylalanine dithiothreitol, 2-mercaptoethanol and [2-amino-2-(hydroxymethyl) propane-1,3-diol] (Tris) (Ruoho et al., 1973; Maasen & Muller, 1974; Beppo et al., 1975; Escher & Schwyser, 1975; Rudnick et al., 1975).

In order not to disturb the binding properties of a natural ligand, it may be necessary to modify a part(s) of the molecule which is not normally involved in recognition and binding. Ideally, a range of ligands, each modified in a different way or place, might be synthesized.

As previously mentioned (p.49) a label with an absorption maximum at wavelengths long enough to be clear of biologically-damaging regions is an advantage. In molecules which are radiolabelled, the labelled atom should be as close to the photolabile group as possible, and not liable to be separated from the rest of the molecule during subsequent manipulations (Bayley & Knowles, 1977).

Bile acid photoaffinity labels

Several different types of bile acid-derived photoaffinity probes have recently been synthesized and used to investigate bile acid-binding proteins (Kramer & Kurz, 1983; von Dippe et al., 1983). These molecules have been employed to study bile salt binding in rat hepatocyte plasma membranes, in rat ileal brush border membrane vesicles, in normal and transformed human and rat hepatocytes, in human serum, and in rat hepatic cytosol (Kramer

et al., 1979; Abberger et al., 1982; Kramer et al., 1982; Burckhardt et al., 1983; Kramer et al., 1983; von Dippe & Levy, 1983). These results have already been discussed.

Among the bile acid-derivatives so far synthesized for use as photoaffinity labels have been both conjugated and unconjugated molecules, with different photolabile groups inserted at a variety of locations within the molecule. These are shown in Figure 1.8, together with specific radioactivities for each molecule, labelled with ^{14}C in the steroid ring structure or ^{3}H in either the steroid ring structure or taurine sidechain, to a low specific radioactivity.

Specific radioactivity Ci/mmol

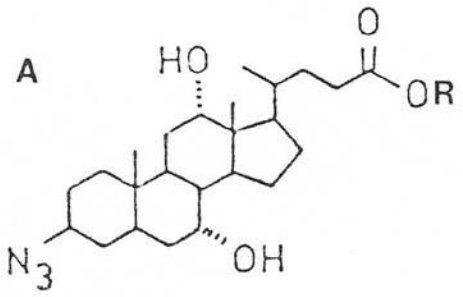
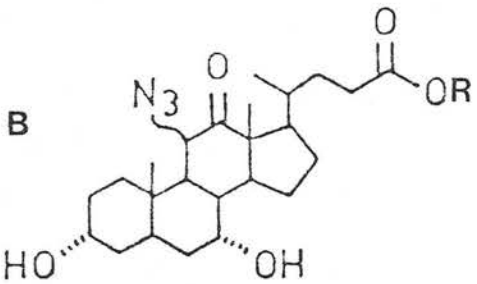
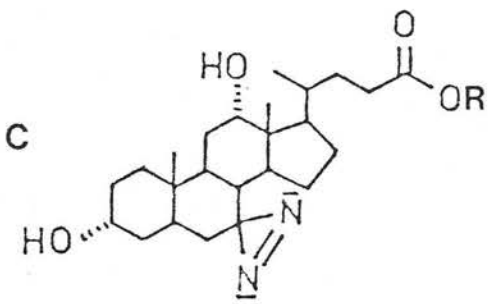
	Nucleus	Sidechain
A 	^{3}H-12β; 2.3	^{3}H-2; 23
B 	^{14}C-24; 50	^{3}H-2; 23
C 	^{3}H-3β, 12β; 3.6 ^{3}H-3β; 2.2	^{3}H-2; 23

FIGURE 1.8 Photolabile bile acid derivatives

(a) 3β-azido-7α,12α-dihydroxy 5β-cholan-24-oic acid (R=H)
(3β-azido-7α,12α-dihydroxy 5β-cholan-24-oyl-2 aminoethanesulphonate (OR=NH.CH_2.CH_2SO_3H)

(b) 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid (R=H)
11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oyl) aminoethanesulphonate (OR=NH.$CH_2CH_2SO_3H$)

(c) 7,7azo-3α,12α-dihydroxy-5β-cholan-24-oic acid (R=H)
(7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulphonate (OR=NH.CH_2.CH_2SO_3H)

OBJECTIVES OF THE THESIS AND SUMMARY OF INVESTIGATIONS

The aims of this study were to synthesize a novel bile acid derivative, 3β-azidocholylhistamine, radioiodinated to a specific radioactivity of approximately 1900 Ci/mmole, and to use this molecule as a photoaffinity probe in the investigation of bile acid-binding proteins in rat hepatic cytosol.

In particular, the newly discovered Y' group of bile acid-binding proteins were studied; along with several glutathione S-transferases, these proteins were analysed for bile acid-binding using the photoaffinity label described above.

As a result of an improved purification scheme, several previously undescribed bile acid-binding proteins were found in the Y' fraction. These fell into two categories:-

(1) Proteins, which were dimers of 15 000 Da - 19 000 Da molecular mass, and which showed a highly specific binding of the photoaffinity label, and were present in small amounts.

(2) Proteins, which were of molecular mass 33 000 Da - 36 000 Da, and which showed a lower specific binding of [^{125}I]-3βazidocholylhistamine. However, as these proteins were present in larger amounts, they contributed as much, if not more, to the total bile acid-binding capacity of the Y' fraction than those

proteins in Group 1.

The binding sites of these proteins, and of the glutathione S-transferases, were investigated by photoaffinity labelling and peptide "mapping" on reverse-phase high pressure liquid chromatography.

MATERIALS AND METHODS

MATERIALS

Chemicals

Chemicals, radiochemicals and solvents were obtained from a variety of commercial sources and were of analytical grade or better. A full list of chemicals and suppliers is given in Appendix I, along with any further purification of chemicals which was undertaken.

Buffers

The composition of the buffers used in this study and the temperature at which they were prepared are listed in Table 2.1.

Animals

Wistar rats (250-300 g), fed *ad libitum*, from the Animal Breeding Research Organisation, Bush Estate, Milton Bridge, Midlothian, U.K., were used in the preparation of rat liver cytosol and for the bile duct cannulation experiments.

Buffer	pH	Temperature buffer prepared	Composition
A	74	20°C	10mM sodium phosphate
B	8	4°C	10mM Tris-HCl
C	6.4-5.5	4°C	25mM Histidine HCl
D	4.0-4.8	4°C	Polybuffer 74-HCl 1:8 v/v distilled water)
E	8.9	20°C	375mM Tris-HCl
F	6.8	20°C	125mM Tris-HCl
G	7.4	20°C	20mM sodium phosphate containing 140mM sodium chloride, 250mM sucrose
H	6.7	4°C	10mM potassium phosphate
I	6.7	4°C	500 mM potassium phosphate

Table 2.1 Buffer compositions.

GENERAL ANALYTICAL METHODS

Chromatography of bile acids

Thin layer chromatography (TLC) was carried out on silica gel 60 glass plates (5 cm x 20 cm x 0.25 mm) (Merck-Darmstadt, FRG) or on Kodak chromagram silica gel plastic sheets (20 cm x 20 cm x 0.1 mm) (Eastman Kodak Co., Rochester, NY., U.S.A.). Column chromatography was performed in glass columns (2.5 cm x 70 cm) using silica gel N60 (43-60 um) (Merck-Darmstadt, FRG). The following solvent systems were used for both thin layer and column chromatography:-

1) Ethyl acetate: cyclohexane: acetic acid (23:7:3 v/v/v)
2) Ethyl acetate: cyclohexane: acetic acid (100:40:1 v/v/v)
3) Chloroform: methanol: acetic acid: water (30:10:3:2 v/v/v/v)
4) Trimethyl pentane: isopropylalcohol:acetic acid (120:40:1 v/v/v)
5) Ethyl acetate: toluene: methanol:triethylamine (5:2.5:2.5:1 v/v/v/v)

Bile acids, including radiolabelled bile acids, were shown to be at least 99% pure by TLC before use, chromatography being carried out on glass plates using solvent system 4 for non-radiolabelled bile acids, and for radiolabelled bile acids, solvent system 3 (Kelly & Doisy, 1964).

The purity of non-radiolabelled bile acids was assessed by staining the developed plates with acetic acid/sulphuric acid/anisaldehyde (100:2:1 v/v/v) and scanning the resulting chromatogram with a Vitatron TLD100 Flying Spot densitometer (from Scientific Apparatus, Loughborough, Leics., U.K.) as described by O'Moore & Percy-Robb (1973). The purity of the radiolabelled bile acid samples was assessed by scraping portions (1 cm x 1 cm) from the developed chromatograms and measuring the radioactivity contained in each silica gel slice by liquid scintillation spectrophotometry. In those cases involving histamine derivatives, the TLC plates were also stained with Pauly's Reagent, an equal mixture of 10% sulphuric acid in 1N hydrochloric acid (HCl) and 5% sodium nitrite (by vol., aq) mixed cold and sprayed on to the plates (Albright et al., 1953). This was followed by spraying with 15% sodium carbonate (by vol., aq) which formed the characteristic rose-brown colour with histamine.

Radioactivity counting

For ^{3}H and ^{14}C, this was performed in a Packard Tri-Carb liquid scintillation spectrometer (Model 3255-Technologies Packard, Caversham, Berks., U.K.) using a toluene-triton X 100 (2:1 v/v) based scintillant containing 2,5-diphenyloxazole (PPO), 4 g/l and p-bis(2-(5-phenyloxazolyl)-benzene (POPOP), 0.2 g/l. Samples, either aqueous (100 ul) or solid (silica gel), were added

to the scintillant (5 ml) in polyethylene vials and counted for either 10 min or until a minimum of 10 000 counts were detected. The counting efficiency was determined by counting known amounts of [^{3}H]cholic acid and [^{14}C]lithocholic acid, and was found to be 60% and 85% respectively.

For ^{125}I, a Nuclear Enterprises NE1600 gamma counter (Nuclear Enterprises Limited, Sighthill Industrial Estate, Edinburgh, U.K.) was used. Samples were counted for a minimum of 10 000 counts.

Equilibrium dialysis

The Dianorm equilibrium dialysis equipment used was obtained from Fisons Ltd., Crawley, Sussex, U.K. The binding of 3β-azidocholic acid to bovine serum albumin was investigated at 20°C in cells each of which consisted of two teflon half-cells separated by a semi-permeable membrane of 32/32 visking tubing. The BSA was charcoal-stripped to remove endogenous bile acids (Simmonds et al., 1973).

The dialysis experiment was run in duplicate. In the left-hand side of one pair of cells was placed 1 ml of 10mM sodium phosphate pH 7.4 (buffer A) containing 0.5 mg 3β azidocholic acid (4.5 nCi), which had been synthesized from a starting material which included tracer amounts of 2,4 [^{3}H]cholic acid. On the right-hand side was placed buffer A (1 ml) only. This pair of cells served as a

control. The second pair of cells was identical to the first except that the right-hand side also contained stripped BSA (20 mg/cell).

The dialysis cells were rotated at 12 rpm for a total of 24 h. At timed intervals a sample (50 µl) was withdrawn from each side of both pairs of cells, and once scintillant (5 ml) had been added, was counted for ^{3}H.

Protein determinations

Protein concentrations in samples eluted from columns were calculated from the extinction values at 280 nm. All other protein determinations were by the method of Bradford (1976) with bovine serum albumin as calibration standard.

Glutathione S-transferase activity

This was measured at 37°C in a Aminco Rotochem IIa parallel fast analyser (American Instrument Co., Maryland, U.S.A.). The conjugation of glutathione with 1-chloro-2, 4-dinitrobenzene was followed at 340 nm in 100mM sodium phosphate, pH 7.4. Reaction rates were corrected for non-enzymic conjugation.

Discontinuous SDS/polyacrylamide-gel electrophoresis

This was performed at room temperature (20°C) in the presence of 0.1% (w/v) sodium dodecyl sulphate (SDS) using vertical slab gels (1.5 mm x 140 mm x 195 mm) in an IN/96 electrophoresis apparatus (Raven Scientific Ltd., Haverhill, Suffolk, U.K.). The electrophoretic system

used was that of Laemmli (1970). The resolving gel was 14 cm long, of either 12% or 16.5% (w/v) polyacrylamide containing 375mM tris-HCl, pH 8.9 (buffer E), and a 1.2 cm high stacking gel of 3.6% w/v polyacrylamide containing 125mM tris HCl, pH 6.8 (buffer F) which was formed on top of the resolving gel.

Molecular mass calibration of the gels was achieved using either a seven protein marker as standards (Figure 2.1) or alternatively a four protein marker containing bovine serum albumin (66 000 Da), ovalbumin (45 000 Da), chymotrypsinogen A (24 000 Da) and myoglobin (17 000 Da).

In those experiments where the gels were sliced, they were cut into 0.5 cm or 0.25 cm slices, and counted for [^{125}I].

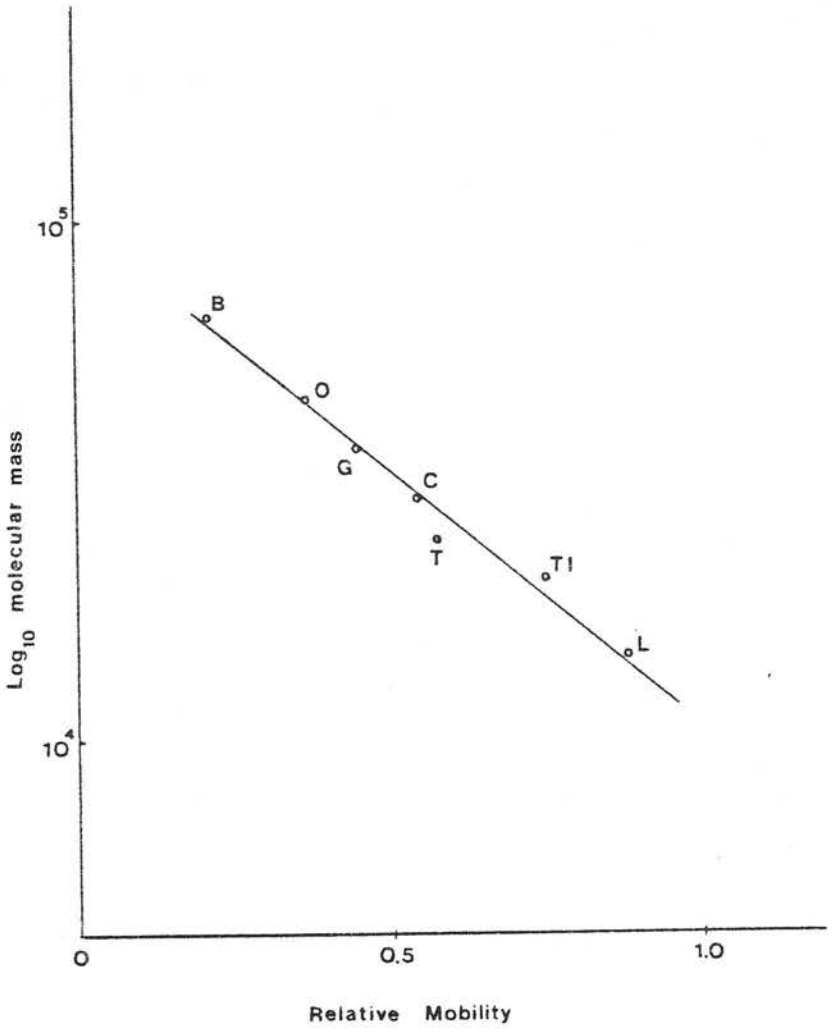

10^5
10^4
Log_{10} molecular mass
B
O
G
C
T
T1
L
0
0.5
1.0
Relative Mobility

FIGURE 2.1 Calibration of SDS/polyacrylamide gel.

Plot of molecular mass of marker proteins against their electrophoretic mobility relative to bromophenol blue on an SDS/polyacrylamide gel (12% resolving gel).

5 ug each of Bovine serum albumin (B; 66 000 Da); Ovalbumin (O; 45 000 Da); Glyceraldehyde-3-phosphate dehydrogenase (G; 36 000 Da); Carbonic anhydrase (C; 29 000 Da); Trypsinogen (T; 24 000 Da); Trypsin inhibitor (TI; 20 000 Da); α-lactalbumin (L; 14 200 Da).

SYNTHESIS AND CHARACTERISATION OF PHOTOAFFINITY LABEL

Synthesis of [^{125}I]-3βazidocholylhistamine

This was performed in two steps -

1) introduction of an azido($-N_3$) group at the 3β--position of cholic acid, essentially according to Kramer & Kurz (1983);

2) conjugation of this bile acid-derivative with histamine and subsequent iodination. Following the method of Beckett et al., (1979); Figure 2.2 shows the overall reaction pathway for the synthesis.

1) Introduction of azido group

A solution of ρ-toluene sulphonyl chloride (5 mmol) in absolute pyridine (2 ml) was added (over 30 min, 0°C with stirring), to a solution of cholic acid (5 mmol) in absolute pyridine (10 ml), also containing 2,4-[^{3}H]-cholic acid (0.1 μCi, 6.5 μmol) as tracer. The solution was warmed and stirred at room temperature for 3 h, the progress of the reaction being monitored by TLC in solvent system 1. When complete, the reaction mixture was added slowly, with stirring, to 1.5M hydrochloric acid (400 ml), the precipitate collected by filtration, and dried in vacuo over silica crystals. This material was dissolved in solvent system 1 and further purified by column chromatography in the same solvent system; 1.038 g of 3α-toluenesulphonyl cholic acid was recovered (1.85 mmol, 40% yield). The

derivative was analysed by TLC (solvent systems 1 and 2) and infra-red spectrophotometry.

The 3α-toluenesulphonyl cholic acid (1.85 mmol) was dissolved with stirring in a warm solution of sodium azide (15.5 mmol) in dry dimethyl sulphoxide (23 ml) and the resulting solution kept in the dark at 100°C for approximately 5 h. All further work was carried out in dim or red light. Reaction progress was monitored by TLC in solvent system 1 and, when complete, the solution was poured with stirring into 1M hydrochloric acid (500 ml). The resulting precipitate was collected by filtration and dried in vacuo over silica crystals. The products were further purified by column chromatography in solvent system 2. The final product was 3β-azidocholic acid (0.7 mmol, 40% yield) and its identity was verified by analysis on TLC (solvent systems 1 and 2), melting point determination, ultraviolet and infra-red spectrophotometry, and ^{1}H and ^{13}C nuclear magnetic resonance spectrometry.

2) Conjugation of 3β-azidocholic acid with histamine

3β-azidocholic acid (0.25 mmol) and N-hydroxysuccinimide (0.275 mmol) were dissolved in sodium-dried tetrahydrofuran (3 ml) and cooled in ice. Dicyclohexyl carbodiimide (0.275 mmol) in sodium dried-tetrahydrofuran (0.5 ml) was added and the mixture stirred at 0°C for 1 h, and then at room temperature for 3 h. Following the addition of a single drop (100 μl) of glacial acetic

NOTE: Exercise extreme caution when handling sodium azide or azide derivatives as these compounds are not only highly toxic, but also potentially explosive.

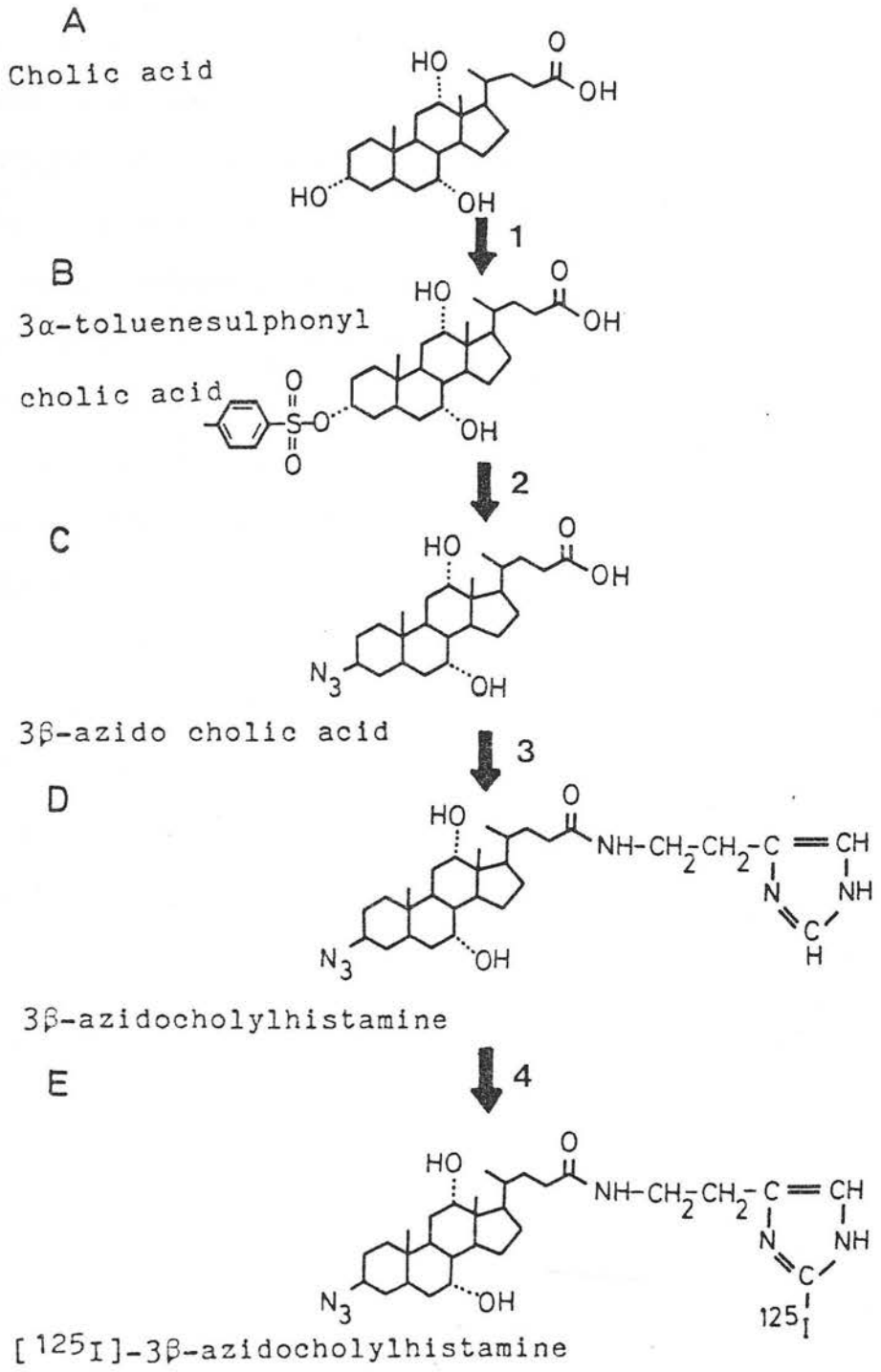


FIGURE 2.2 Reaction pathway for synthesis of [^{125}I]-3βazidocholylhistamine.

1,2 - introduction of azido group.

3,4 - conjugation with histamine and subsequent iodination.

acid, the mixture was stirred for a further 1 h. After filtration, the precipitate was washed with ethyl acetate, the washings and filtrate combined and taken to dryness on a rotary evaporator at approximately 60°C. The residue was dissolved in dimethylformamide (3 ml) and histamine (0.275 mmol) added. The mixture was stirred overnight at room temperature. Using a rotary evaporator at approximately 95°C, dimethylformamide was removed and the residue dissolved in ethyl acetate (1 ml) at approximately 50°C with stirring and dropwise addition of methanol. After cooling the solution, any crystals of bile acid and urea which immediately formed were removed by centrifugation. The remaining solution was evaporated at room temperature under nitrogen to approximately 0.5 ml and applied to two silica gel plates, which were developed in solvent system 3. The histamine conjugate was visualised by spraying the two margins of the plate with anisaldehyde solution, and the area of the gel containing the product was scraped from the plate, eluted with methanol, evaporated at room temperature under nitrogen, weighed and re-dissolved in methanol to produce a 5 mmol/l solution. The yield of 3β-azidocholylhistamine was 50%, and it was analysed on TLC in solvent systems 1 and 2.

The conjugate (50 mmol, 10 μl) was mixed with [^{125}I]-sodium iodide (1 mCi, 10 μl) and a solution of chloramine T (0.18 μmol, 10 μl) in buffer A, for 20 sec.

The reaction was terminated by the addition of sodium metabisulphite (1.6 μmol, 10 μl) in water. The solution was extracted with ethyl acetate (0.2 ml), the organic layer removed and dried with anhydrous sodium sulphate. The radiolabelled conjugate was then separated from unreacted [^{125}I]sodium iodide and non-iodinated conjugate by TLC in solvent system 3 on plastic plates [^{125}I]-3β-azidocholylhistamine was located on the plate using a scanning radiation counter and eluted from the silica gel by ethanol. Radioactive incorporation was 30-50%. The conjugate was stored in the dark at 4°C, having a specific radioactivity of approximately 1900 Ci/mmol. [^{125}I]-3β-azidocholylhistamine was run on TLC in solvent systems 1 and 2, and a "cold" iodination performed on a 10^3 times larger scale in order to generate an iodinated conjugate for infra-red spectrophotometry.

Hepatic transport of [^{125}I]-3β-azidocholylhistamine

Female Wistar rats were anaesthetised with diethyl ether and the common bile duct cannulated with nylon tubing (0.1 mm i.d.). The test solutions were injected into the superior mesenteric vein in 150mM saline (75 μl) containing approximately 20% ethanol by volume. Bile was collected at 1 min intervals in pre-weighed tubes and the radioactivity counted. In experiments using the [^{125}I]-labelled derivative, the bile samples were counted directly whereas ^{14}C-labelled bile acids were counted

following the addition of scintillant. After extraction with ethanol, bile samples were run on TLC, solvent system 3, and the plates scraped to locate the radioactivity.

Gel filtration of rat hepatic cytosol incubated with radiolabelled bile acids

A portion of dialysed rat hepatic cytosol (5 ml, 100 mg) in buffer A was incubated with either (carboxy-^{14}C) lithocholic acid (1.5 μCi) or [^{125}I]-3βazidocholylhistamine (5 μCi) at 4°C for 30 min. and, in the latter case, in the dark. The samples were then applied separately to a column of Sephadex G-75 superfine (2.6cm x 96cm) and eluted with buffer A. Fractions were analysed for protein concentration, glutathione S-transferase activity, [^{14}C] and [^{125}I].

PHOTOLYSIS REACTOR AND PHOTOAFFINITY LABELLING

Photoaffinity labelling

Photolysis experiments were carried out in an annular photoreactor, (Applied Photophysics Ltd., London, U.K.), Figure 2.3, with a 400 watt medium pressure mercury arc lamp. This has an output of >5 x 10^{19} photons/sec, as measured by ferrioxalate actinometry, and radiates predominantly at 313 nm and 365 nm. The lamp was water-cooled, and a borosilicate glass filter with wavelength cut-off of approximately 300 nm was employed. Test solutions (8-10 ml) were held in quartz tubes (11 mm i.d.) with a glass rod (7 mm o.d.) located centrally to give a thin film of approximately 2 mm. The tubes were rotated at 25 revolutions per minute around the light source.

The iodinated photoaffinity label, proteins, or a mixture of protein with the label, were photolysed in either buffer A or B (8-10 ml) following incubation in the dark at 20°C for 15 min. The protein concentration ranged from 50 µg/ml to 0.5 mg/ml, and the concentration of [^{125}I]-3β-azidocholylhistamine from 1.25 pmol (2.5 µCi) to 5 pmol (10 µCi). The duration of photolysis varied from 15 min to 60 min. At timed intervals the solutions were mixed and, if relevant, portions were removed for analysis. All subsequent handling was done in the dark or under red light.

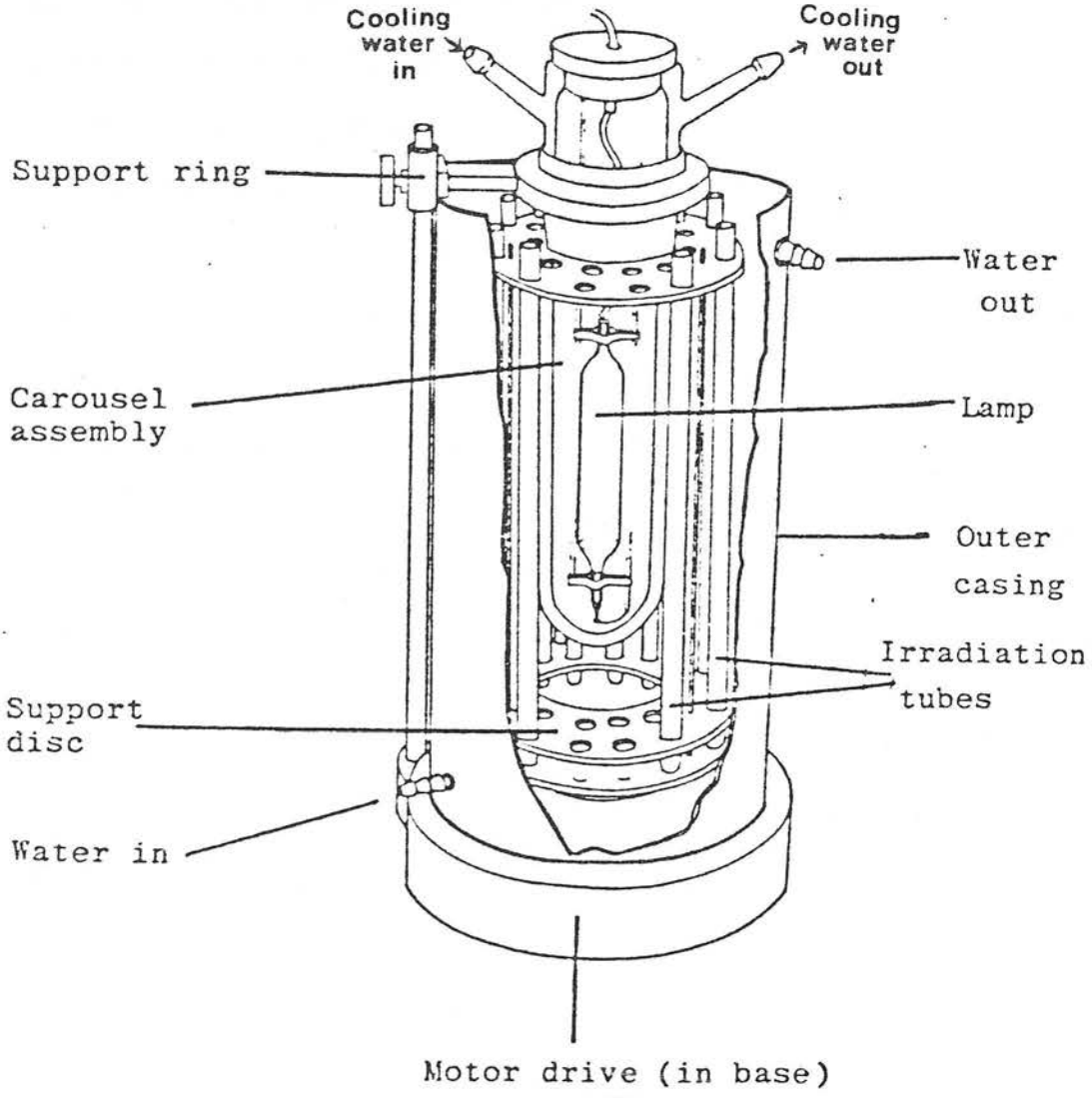


FIGURE 2.3 Annular photoreactor (Applied Photophysics, Ltd).

Analysis of labelling

After photolysis, the protein solutions (or portions of them) were precipitated by adding ice-cold trichloroacetic acid (TCA) to a final concentration of 10% by volume. After 30 min on ice, the samples were centrifuged (MSE mistral 6L, MSE Scientific Instruments, Crawley, Sussex, U.K.), at 1500 g, 4°C for 45 minutes. The supernatant was decanted, and the precipitates washed three times, each with 5 ml of 10% TCA, by the same procedure. The precipitates were then either taken up in the appropriate mixture and prepared for SDS/polyacrylamide-gel electrophoresis (see p.65) or taken up in buffer A containing 1% SDS. The protein solutions in the latter case were then applied to a 1 cm x 20 cm column of Sephadex G-25 fine, equilibrated with buffer A containing 1% SDS, and eluted (25 ml/h) in the dark with the same buffer. Fractions of 4 ml were collected and analysed for [^{125}I].

Alternatively, the photolysed protein solutions (or portions of them) were applied directly to a column (2.6 cm x 42 cm) of Sephadex G-100 fine, equilibrated in buffer A. The labelled protein was eluted with buffer A.

"Peptide mapping" was performed on photoaffinity labelled Y and Y' proteins using reverse-phase high pressure liquid chromatography. Details are given later in this section (p. 84).

PURIFICATION OF Y' PROTEINS

Purification of Y' proteins

This was done esssentially as in Sugiyama et al. (1982, 1983) with important modifications which resulted in the discovery of previously unknown bile acid-binding proteins. These will be discussed in a later section.

Table 2.2 gives the details of the columns used at each stage of the purification, and Table 2.3 shows a flow diagram of the purification scheme.

Appendix III gives details of the preparation of rat hepatic cytosol.

Cytosol, from eight rat livers, was dialysed (16 h, 4°C) against two changes, each of two litres, of buffer A. The dialysed solution was eluted (25 ml/h) from two columns of Sephadex G-100 fine, connected in series, and equilibrated with buffer A. Those fractions containing glutathione S-transferase activity were pooled and concentrated to approximately 90 ml by ultrafiltration in a Millipore Ultrafiltration cell (Millipore (UK) Ltd., Harrow, Middlesex, U.K.) using oxygen-free nitrogen at a pressure of 40 pounds per square inch, and PT series membrane (Molecular mass cut-off approximately 10 000 Da).

The concentrated protein solution was eluted (15 ml/h) from two columns of Sephadex G-75 superfine, connected in series and equilibrated with buffer A. Once again, fractions were analysed for protein concentration and glu-

STEP	MATRIX	COLUMN SIZE	BREAKTHROUGH VOID/VOL.(ml)	TOTAL BED VOL. (ml)
Gel filtration I	Sephadex G-100 fine	Two, in series, each 4.4 cm x 88 cm	1000	2675
Gel filtration II	Sephadex G-75 superfine	Two, in series, 3.6 cm x 82 cm and 4.4 cm x 88 cm	700	2175
Gel filtration III	Sephadex G-75 superfine	Two, in series, 3.6 cm x 82 cm and 4.4 cm x 88 cm	700	2175
Ion-Exchange	DEAE-A50 Sephadex	2.2 cm x 25 cm	90	95
Chromatofocusing	Poly buffer exchanger 94	1 cm x 28 cm	20	22
Hydroxyapatite	Biogel HT	2.2 cm x 15 cm	45	55

TABLE 2.2 Details of columns used at each step in the purification of Y' proteins.

Rat livers (8) removed in buffer G (ice cold), chopped finely and homogenised.

Homogenate centrifuged 18,000 g, 4°C for 30 min, and supernatant recentrifuged 100,000 g, 4°C for 60 min.

Supernatant filtered through glass wool plug, then dialysed against 2 changes, each of 2 litres, of buffer A for 18 h.

Dialysed material eluted from Sephadex G-100 fine equilibrated with buffer A. Fractions with glutathione S-transferase activity pooled and concentrated by ultrafiltration.

Concentrated material eluted from Sephadex G-75 superfine equilibrated with buffer A. Fractions with Y' proteins on SDS/PAGE pooled and concentrated by ultrafiltration.

Concentrated material eluted from Sephadex G-75 superfine equilibrated with buffer A. Fraction with Y' proteins pooled, dialysed against 2 changes, each of 2 litres, of buffer B for 18 h.

Dialysed material applied to DEAE-Sephadex A-50 equilibrated with buffer B. After washing with buffer B (100 ml), eluted with 0-200mM linear NaCl gradient in buffer B, than two steps of 500mM and 1M NaCl.

Peaks 5,6,7 and 8 pooled, dialysed against 2 changes, each of 1 litre, of buffer C for 12 h. Applied to PBE 94, equilibrated with buffer C, and eluted with buffer D.

Further purification of bile acid-binding proteins on chromatofocusing or hydroxyapatite as required.

FIGURE 2.3 Purification scheme for Y' proteins

tathione S-transferase activity. Portions of the fractions across the enzyme peak were run on SDS/PAGE. From these results, fractions from the latter half of the glutathione S-transferase peak were pooled and concentrated by ultrafiltration to approximately 40 ml.

This solution was re-applied to the columns of Sephadex G-75 superfine and eluted with buffer A. Analysis of the fractions collected revealed a distinct protein peak which eluted just after the eluate with glutathione S-transferase activity (i.e. Y peak). Portions of the fractions from both peaks (Y and Y') were analysed by SDS/polyacrylamide electrophoresis, and the Y' - containing fractions were combined on the basis of these results so as to minimise cross-contamination of Y and Y' .

The Y' peak was dialysed (16 h, 4°C) against two changes, each of two litres of 10mM tris-HCl, pH 8 (buffer B), before being eluted from a column of diethylaminoethyl (DEAE) Sephadex A-50 equilibrated with buffer B. After 300 ml had been collected, protein which had been retained on the column was eluted with a linear 0-200mM sodium chloride gradient in buffer B, followed by two steps of 500mM and 1M sodium chloride, both in buffer B.

Each of the protein peaks resolved from the ion-exchange column were pooled and subjected to photoaffinity labelling to determine which possessed bile acid-binding

activity. Peaks 5, 6, 7 and 8 were then dialysed (12 h, 4°C) against two changes, each of 1 litre, of 25mM histidine-HCl (buffer C), and concentrated to approximately 10 ml by ultrafiltration. Each was then subjected to chromatofocusing on columns of PBE 94 equilibrated with buffer C. Each column was "primed" with approximately 5 ml of polybuffer 74-HCl (buffer D) before application of the protein sample. The proteins were then eluted with buffer D (200 ml) over a pH gradient of approximately 6.4 to 4. The fractions were analysed for protein concentration and, also pH, using a Radiometer PHM 64 Research pH meter (Radiometer, Copenhagen, Denmark). Portions of the resultant peaks from chromatofocusing were run on SDS/PAGE, and subjected to photoaffinity labelling to test for bile acid-binding.

Those protein peaks from chromatofocusing which gave a high concentration of radioactive bile acid after photoaffinity labelling were further purified, by either chromatofocusing (pH 6.0 - 5.0) or applications to a column of hydroxyapatite, equilibrated with 10mM potassium phosphate, pH 6.7 (buffer H), and elution with a linear gradient (200 ml) of 500mM potassium phosphate, pH 6.7 (buffer I).

PEPTIDE "MAPPING" OF BILE ACID-BINDING PROTEINS

Y' proteins by limited proteolytic digestion in the presence of SDS

The method used was that of Cleveland et al. (1977). The purified bile acid-binding proteins from the Y' fraction after chromatofocusing were precipitated by addition of ice-cold TCA to a final concentration of 10% (w/v) and kept at 4°C for 30 min. The proteins were centrifuged for 45 min at (1500 g), 4°C, and the supernatant decanted. The precipitates were washed twice in a similar manner with ice-cold TCA (10% w/v, 2ml) before being re-dissolved at a concentration of approximately 0.5 mg/ml in a sample buffer containing buffer F, 0.5% SDS, 10% sucrose, and 0.2% bromophenol blue. After heating to 100°C for 2 min, the samples were cooled and α-chymotrypsin added to a final concentration of 100 μg/ml, 12.5 μg/ml or 2.5 μg/ml. Digestion was then carried out at 37°C for 60 min. Proteolysis was stopped following the addition of 2-mercaptoethanol and SDS to final concentration of 10% and 2% (v/v and w/v) respectively, by boiling the samples for 2 min. A portion of each sample (approximately 20 μg) was loaded into a sample well of a 16.5% SDS/polyacrylamide gel, and the gel was run in the normal manner. Undigested samples of each protein, and also a sample of α-chymotrypsin, were included on the gels for reference, along with a marker consisting of seven proteins as standards (Figure 2.1).

Y' proteins and glutathione S-transferases by tryptic digestion and analysis on reverse-phase high performance liquid chromatography

Portions (0.5 - 1.0 mg) of each bile acid-binding protein identified and purified from the Y' fraction were desiccated to dryness under vacuum and then oxidised on ice with performic acid for 2 h. The oxidised proteins were desiccated to dryness under vacuum once more and taken up in 0.2M ammonium acetate, a pH of 8.5 being achieved with 0.2M ammonia. Digestion was carried out at 37°C for 16 h using diphenyl carbamyl chloride-treated trypsin; a protein:protease ratio of 50:1 was employed. The digested proteins were desiccated to dryness under vacuum and then dissolved in 0.1% (v/v) trifluoroacetic acid.

The soluble tryptic peptides were resolved by reverse-phase high performance liquid chromatography (HPLC) on a Waters C-18 u Bondapak column (0.39 cm x 30 cm) in 0.1% trifluoroacetic acid with a gradient (0 - 60%) of acetonitrile. The eluted peptides were detected by their absorbance at 254 nm. The HPLC system employed two Waters Model 510 HPLC pumps, a Model 680 Automated Gradient Controller, a Model 481 Lambda-Max absorbance detector and a Model U6K Universal injector (Waters Associates (Instruments) Ltd., Northwich, Cheshire).

In those experiments where the Y' proteins and glutathione S-transferases were photoaffinity labelled prior to peptide "mapping", these proteins were TCA precipitated

and extensively washed as previously described (see p. 77) before being oxidised and digested. All handling of labelled proteins was performed under red light or in the dark. The HPLC system was connected to a Waters Model 2211 superrac fraction collector to measure the radioactivity associated with the eluted peptides.

RESULTS

SUMMARY OF EXPERIMENTS

The object of this thesis was to investigate bile acid-binding proteins in rat liver cytosol employing the technique of photoaffinity labelling. A radioiodinated bile acid derivative, incorporating a photoreactive group, was synthesized and used as a photoaffinity probe in the study of such proteins. The molecule [^{125}I]-3βazido-cholylhistamine, was shown to be transported by rat liver in a similar manner to a natural bile acid, and was comprehensively characterized. The recently-discovered Y' group of bile acid-binding proteins was purified and previously undescribed proteins found. The site(s) of binding of bile acids to these proteins was investigated by "peptide mapping" with HPLC. In addition, certain glutathione S-transferases were studied to provide a comparison of bile acid-binding sites.

SYNTHESIS AND CHARACTERIZATION OF PHOTOAFFINITY LABEL

Synthesis of [^{125}I]-3βazidocholylhistamine

A scheme for the synthesis of [^{125}I]-3βazidocholyl-histamine is shown in Figure 3.1. The initial step involves the selective tosylation in pyridine of the 3α-position of cholic acid (Barrett & Reichstein, 1938). If the reaction is allowed to continue for a longer period and/or in the presence of excess p-toluenesulphonyl chloride, di- and tri-tosylate derivatives of cholic acid begin to form. Simply on steric considerations, the 3α-

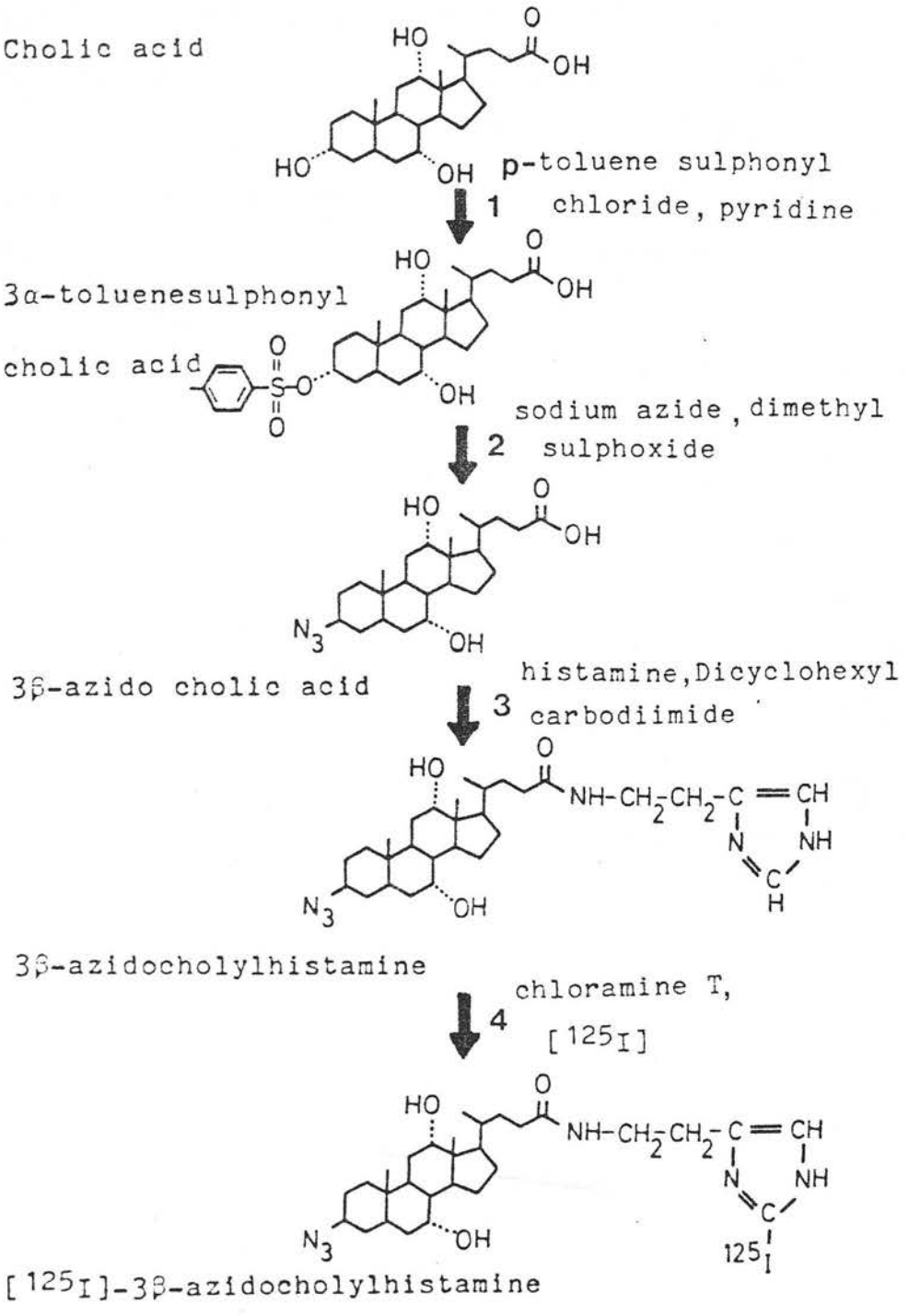


FIGURE 3.1 Synthesis of [^{125}I]-3β-azidocholylhistamine.

-position would be expected to react before the 7α- and that in turn before the 12α-position. The reagents used must be absolutely dry, in order to avoid hydrolysis of the tosyl moiety after formation. Similarly, at the next stage of synthesis, the reaction was performed in absolute dimethysulphoxide in order to protect the tosylate.

Addition of histamine and subsequent iodination was carried out using a well-proven method which is simple and quick to perform (Beckett et al., 1979). The yield of radioiodinated ligand was good, at approximately 25%, with a high specific radioactivity of approximately 1900 Ci/mmol. Good separation of iodinated from non-iodinated derivative is essential if a high specific radioactivity is to be attained. Figure 3.2 demonstrates this point, and illustrates why solvent system 3 was chosen for use in the iodination procedure.

Characterisation

Characterisation of the intermediates and the final product of the synthesis procedure was carried out at each stage. Table 3.1 summarises the relevant information.

Figures 3.3 to 3.6 show the infra-red spectra obtained from the intermediates. The presence of the azido group is demonstrated by the characteristic peak at 2120 cm^{-1} (Figures 3.4, 3.5). The other peaks common to all the intermediates, at approximately 3500 cm^{-1}, 2950 cm^{-1} and 1705 cm^{-1}, are due to O-H, C-H and C=O bond-stretching respectively (Barrow, 1973).

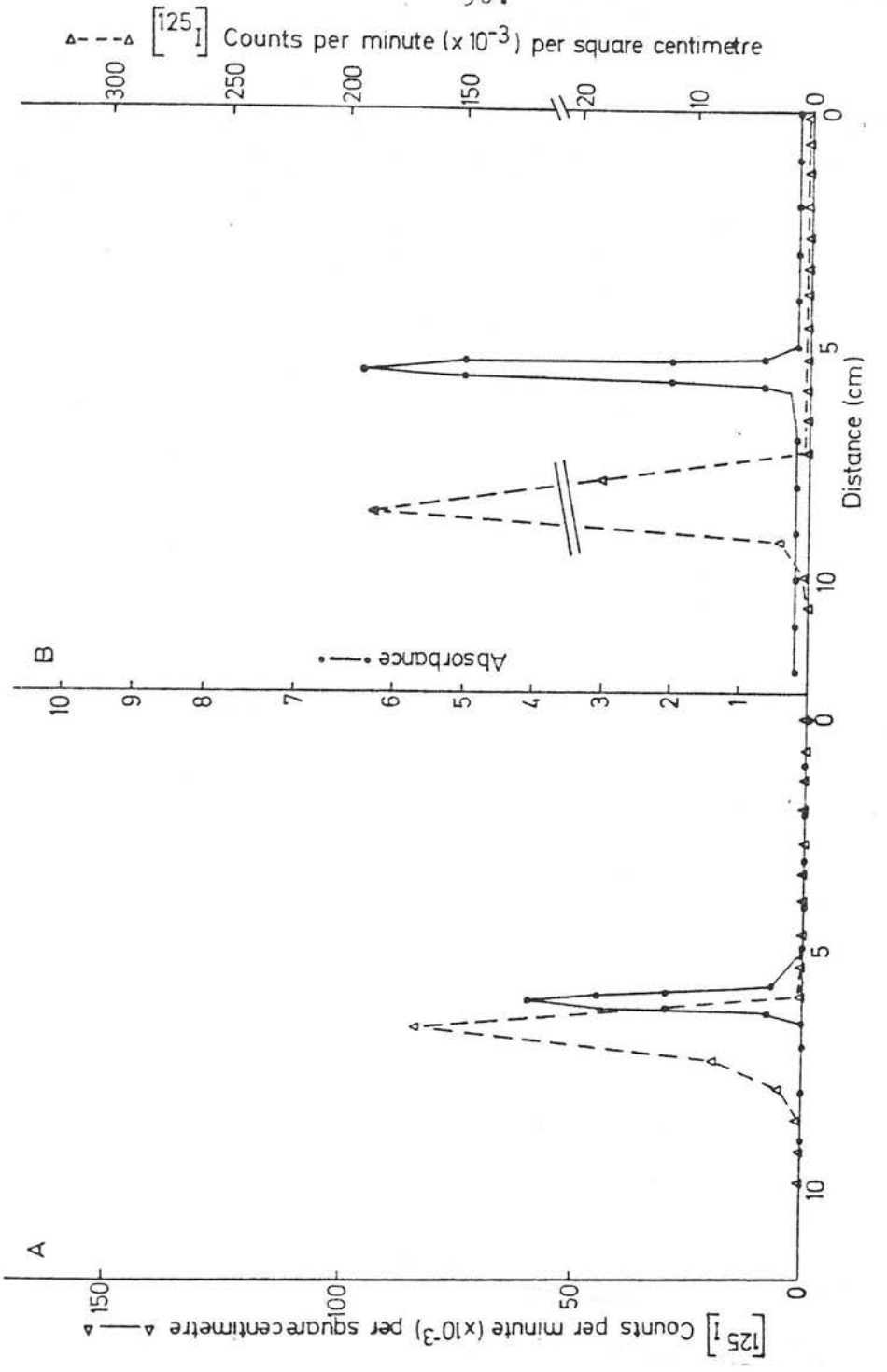


FIGURE 3.2 Separation of [^{125}I]-3βazidocholylhistamine (Δ- - - -Δ) from 3βazidocholylhistamine (•——•) on TLC (0.15 μmol and 0.1 μCi, respectively) in solvent systems 5(A) and 3(B).

Table 3.1. Characterisation of intermediates in the synthesis of [^{125}I]-3βazidocholylhistamine

	MOLECULE	Melting Point	Thin-layer Chromatography	Ultraviolet	Infra-red	NUCLEAR MAGNETIC RESONANCE ^{1}H	NUCLEAR MAGNETIC RESONANCE ^{13}C
A	Cholic acid		0.19/0.02		1705 cm^{-1} (C=0)		
B	3α-toluenesulphonyl cholic acid		0.86/0.54		1705 cm^{-1} (C=0)		
				λ max ϵ		$CDCL_3$ δ =	DMSO-d6 δ =
C	3β-azido cholic acid	209-212°C	0.85/0.39	220nm 262	2105 cm^{-1} (N_3)	0.7(S,CH_3-18)	174.9(CO_2H)
				285 nm 45	1700 cm^{-1} (C=0)	0.92(S,CH_3-19)	TOTAL = 24 C atoms C = 3
						0.97(d,J=6H_3, CH_3-21)	CH = 9 CH_2= 9 CH_3= 3 24
D	3β-azidocholyl-histamine		0.47/0.6		2120 cm^{-1} (N_3) 1580 cm^{-1}		
E	[^{125}I]-3βazidocholyl-histamine		0.86/0.75		2100 cm^{-1} (N_3) 1570 cm^{-1}		

Table 3.1 Characterization of the intermediates in the synthesis of [^{125}I]-3βazidocholylhistamine. TLC figures (A-C) are Rf values in solvent system 1/solvent system 2; D and E are Rfs in solvent system 3/solvent system 5.

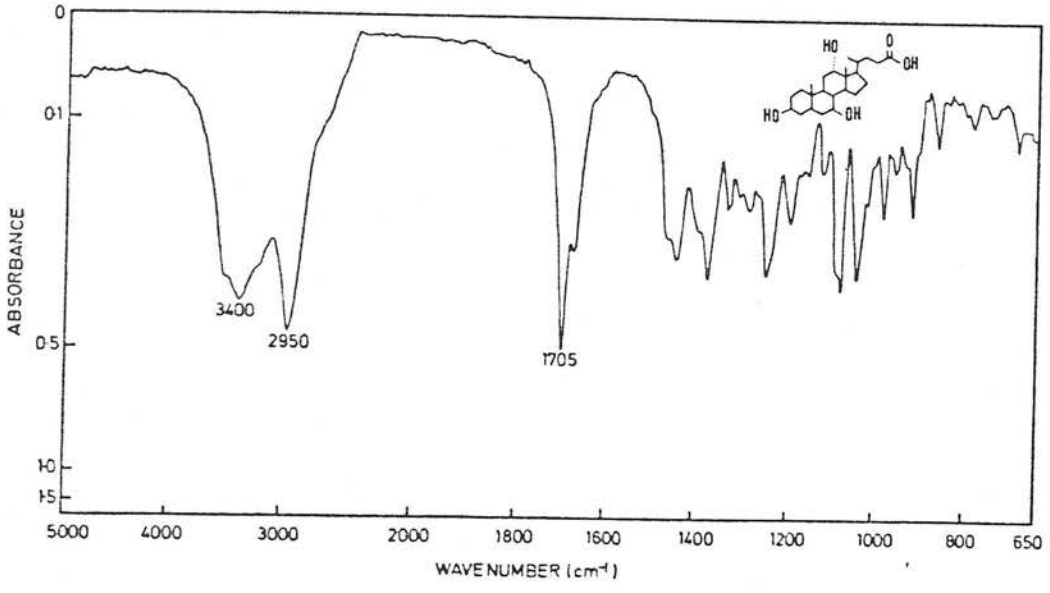


FIGURE 3.3 Infra-red spectrum of cholic acid. Sample was mixed with dry, pure potassium bromide and ground finely before being pressed into a disc (approximately 1 mm thick) under vacuum.

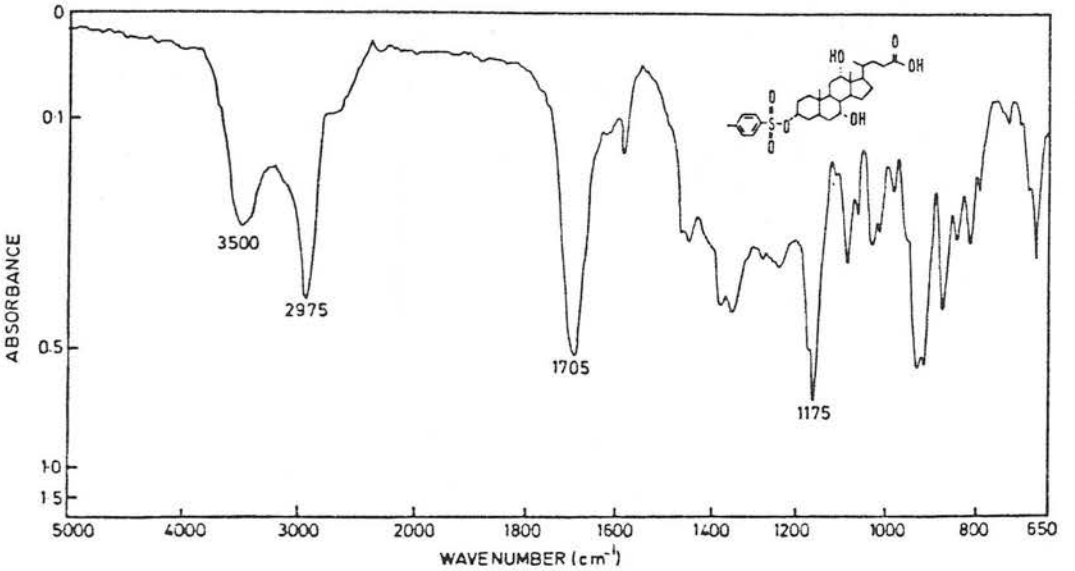


FIGURE 3.4 Infra-red spectrum of 3α-toluenesulphonyl cholic acid (sample preparation as Figure 3.3).

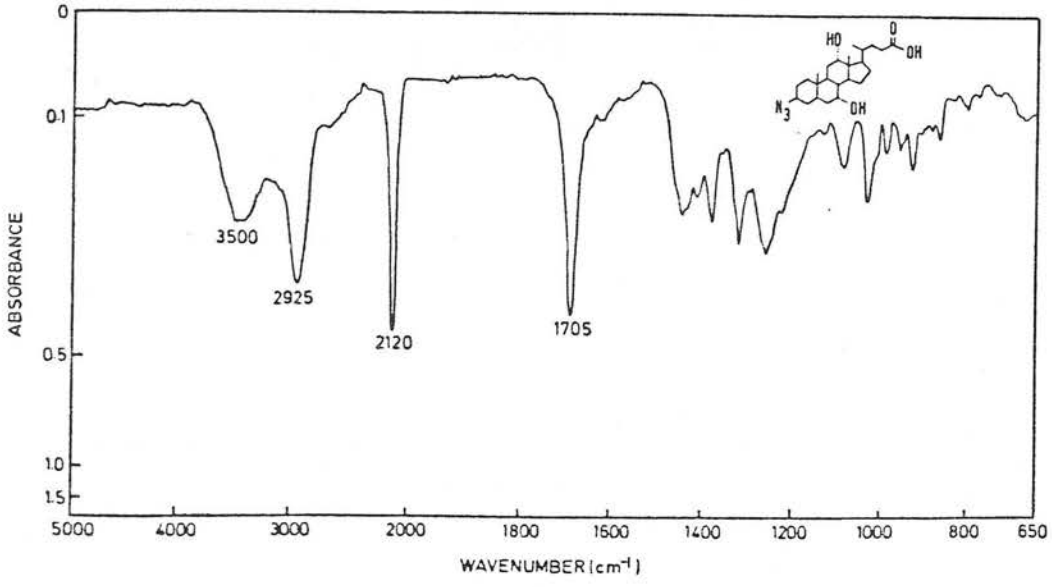


FIGURE 3.5 Infra-red spectrum of 3β-azidocholic acid (sample preparation as Figure 3.3).

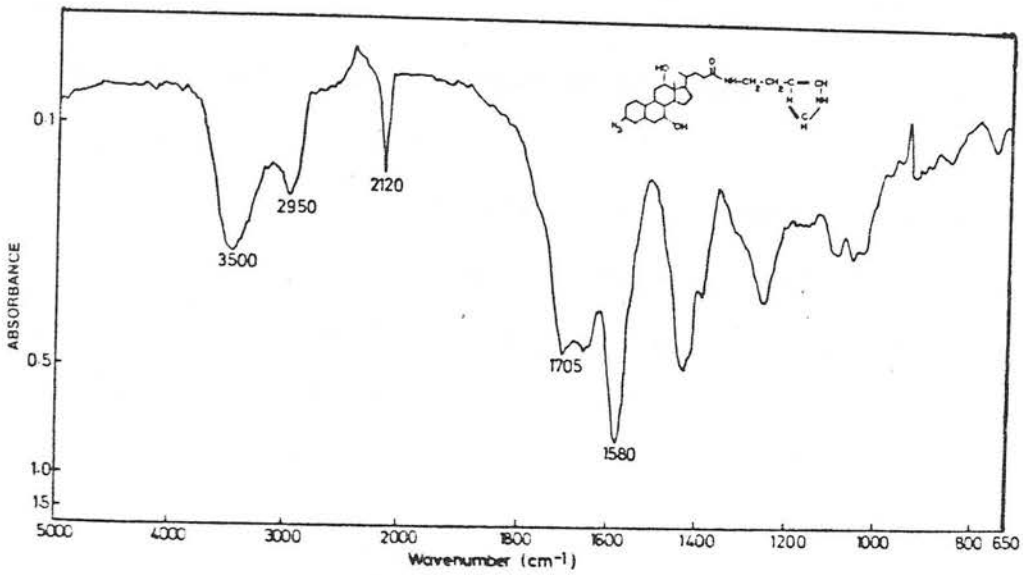


FIGURE 3.6 Infra-red spectrum of 3β-azidocholylhistamine (sample preparation as Figure 3.3).

An infra-red spectrum of [^{125}I]-3βazidochohylhistamine was also carried out, and is essentially the same, in all important features, as that of its immediate precursor (Figure 3.6).

Proton and ^{13}C nuclear magnetic resonance spectroscopy was employed to help confirm the molecular structure of 3β-azidocholic acid. The results of the proton NMR scan (Figure 3.7) were in agreement with that of Kurz & Gerok (1983). ^{13}C NMR confirmed that the molecule did have 24 carbon atoms, with the distinctive carboxyl peak at a high field position (Figure 3.8). In addition, a distortionless enhanced polarisation transfer (DEPT) ^{13}C NMR scan was carried out (Figure 3.9). This technique enables a count of the total number of -CH and -CH_3 groups in the molecule to be made, and also the total number of -CH_2 groups. The results correspond to the predicted figures for 3β-azidocholic acid.

It was demonstrated that bovine serum albumin reversibly bound 3β-azidocholic acid by equilibrium dialysis, and the results are shown in Figure 3.10.

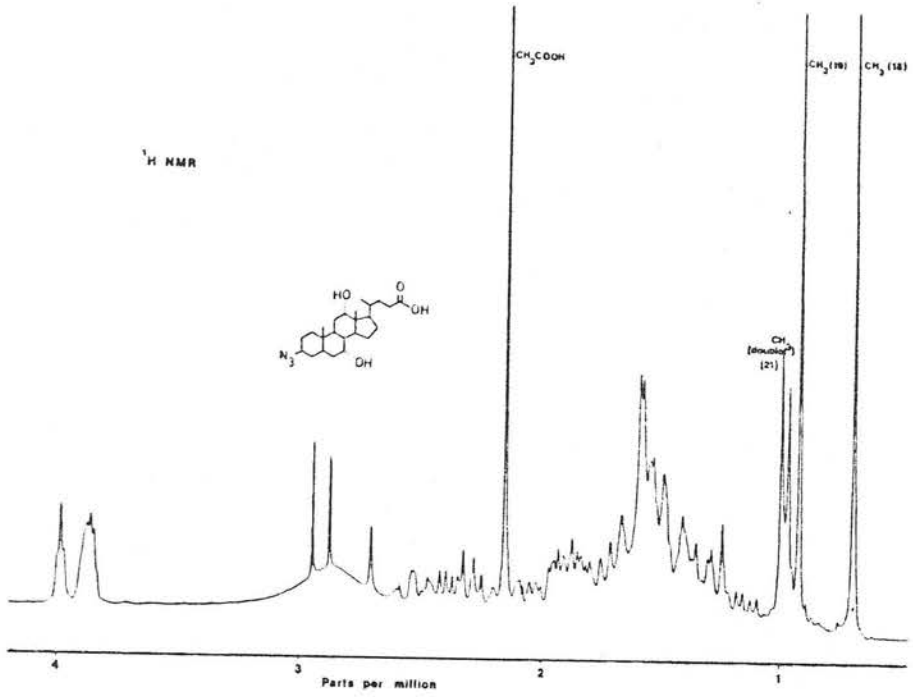


FIGURE 3.7 Proton NMR scan of 3β-azidocholic acid (inset shows structure of molecule).

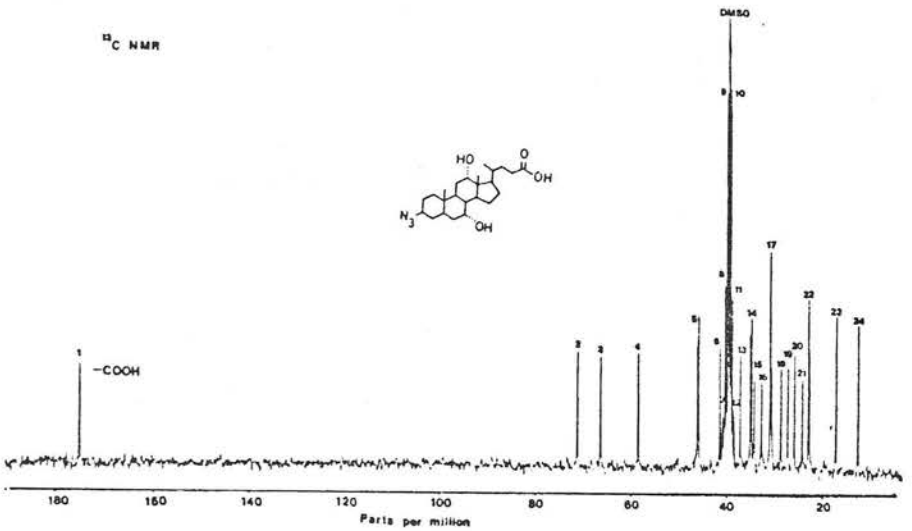


FIGURE 3.8 ^{13}C NMR scan of 3β-azidocholic acid (inset shows structure of molecule). Total number of carbon atoms = 24.

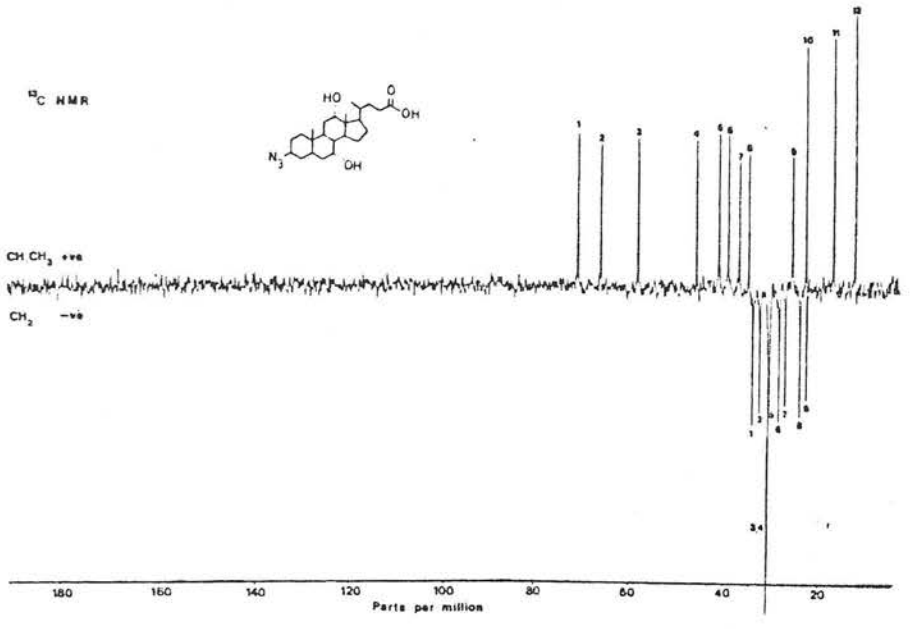


FIGURE 3.9 ^{13}C NMR scan of 3β-azidocholic acid (inset shows structure of molecule). Total -CH/ $-CH_3$ groups = 12; total $-CH_2$ groups = 9).

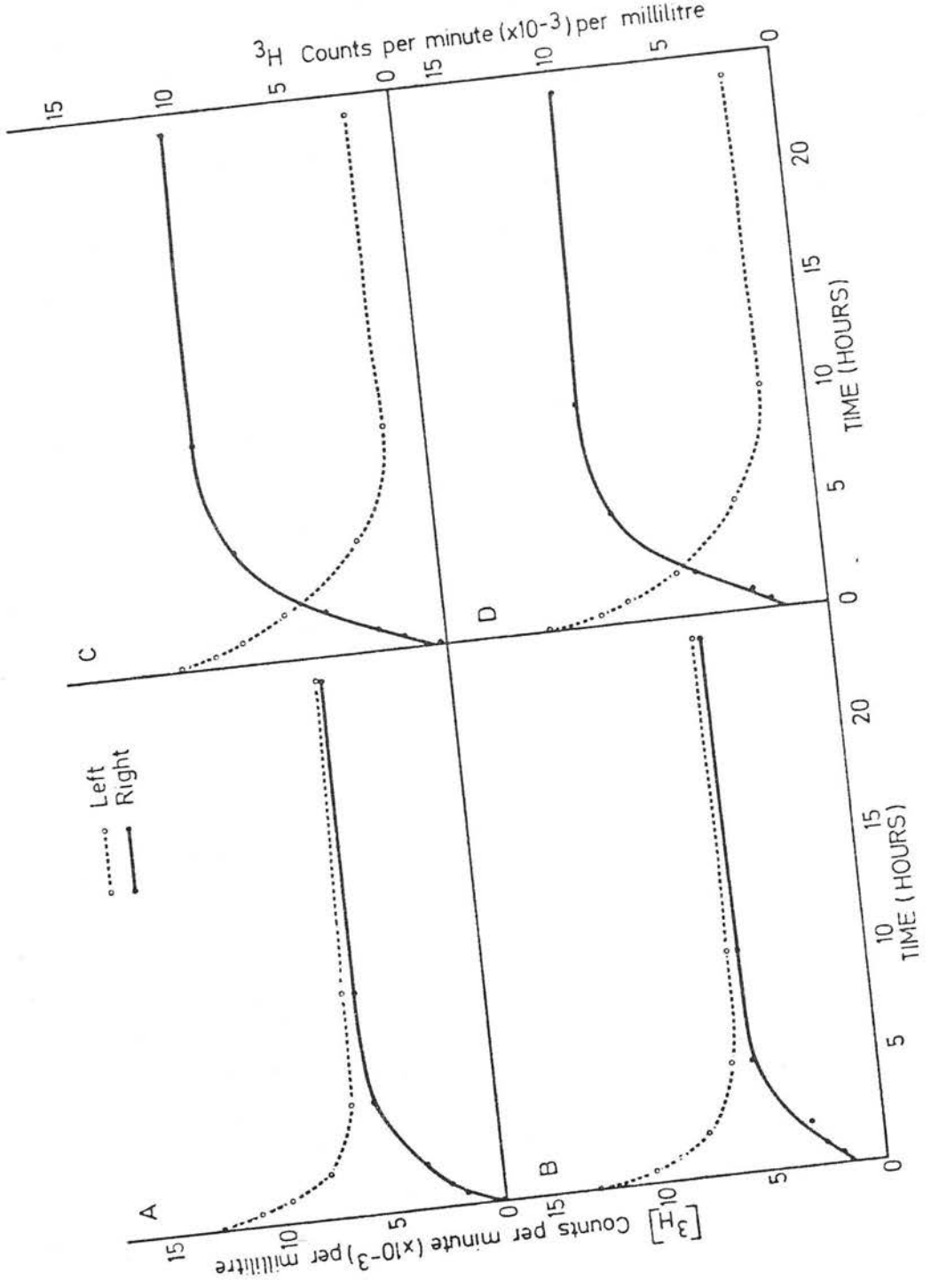

^3H Counts per minute (x10-3) per millilitre
Left
Right
A
B
C
D
TIME (HOURS)
TIME (HOURS)

FIGURE 3.10 Investigation by equilibrium dialysis of the binding of 3β-azidocholic acid to bovine serum albumin. A,B = controls; C,D = BSA on right side of cells.
3β-azidocholic acid (0.5 mg) with trace amounts of 2,4[^{3}H]-label, was placed on the left side of each cell. Dialysis was carried out at 20°C for 24 h, with portions of 50 µl being removed from both sides of each cell, at timed intervals, for counting.

Hepatic transport of [^{125}I]-3βazidocholylhistamine

In order to assess the capacity of rat liver to handle the photoaffinity label, the molecule was injected into the hepatic portal system via the superior mesenteric vein, and bile collected from a cannula. Figure 3.11 shows the results of an experiment in which [^{125}I]-3βazidocholylhistamine only was injected, the test being performed in duplicate. Radioactivity first appeared in bile after 3 min, and reached a peak at 8 min, thereafter falling away to zero after approximately 60 min. The results from both rats were virtually identical, as can be seen from the figure. Bile flow was also measured, and showed no appreciable change during the course of the experiment.

To obtain a comparison with a natural bile acid [^{125}I]-3βazidocholylhistamine* was injected together with [^{14}C]-taurocholate, and bile collected. Figure 3.12 shows that both bile acids rapidly appeared in bile, although taurocholate peaked 2 min earlier than the photoaffinity label. Apart from this difference, the excretion profiles of the two molecules were essentially the same, suggesting that [^{125}I]-3βazidocholylhistamine was handled efficiently by rat liver.

*NOTE: Investigation of bile acid transport using [^{125}I]-cholylglycltyrosine, which has a net negative charge, has demonstrated the relatively inefficient uptake of neutral molecules such as [^{125}I]-3β -azidocholylhistamine. (Spenney et al., 1979; van Dyke et al., 1982; Suchy et al., 1982, 1983).

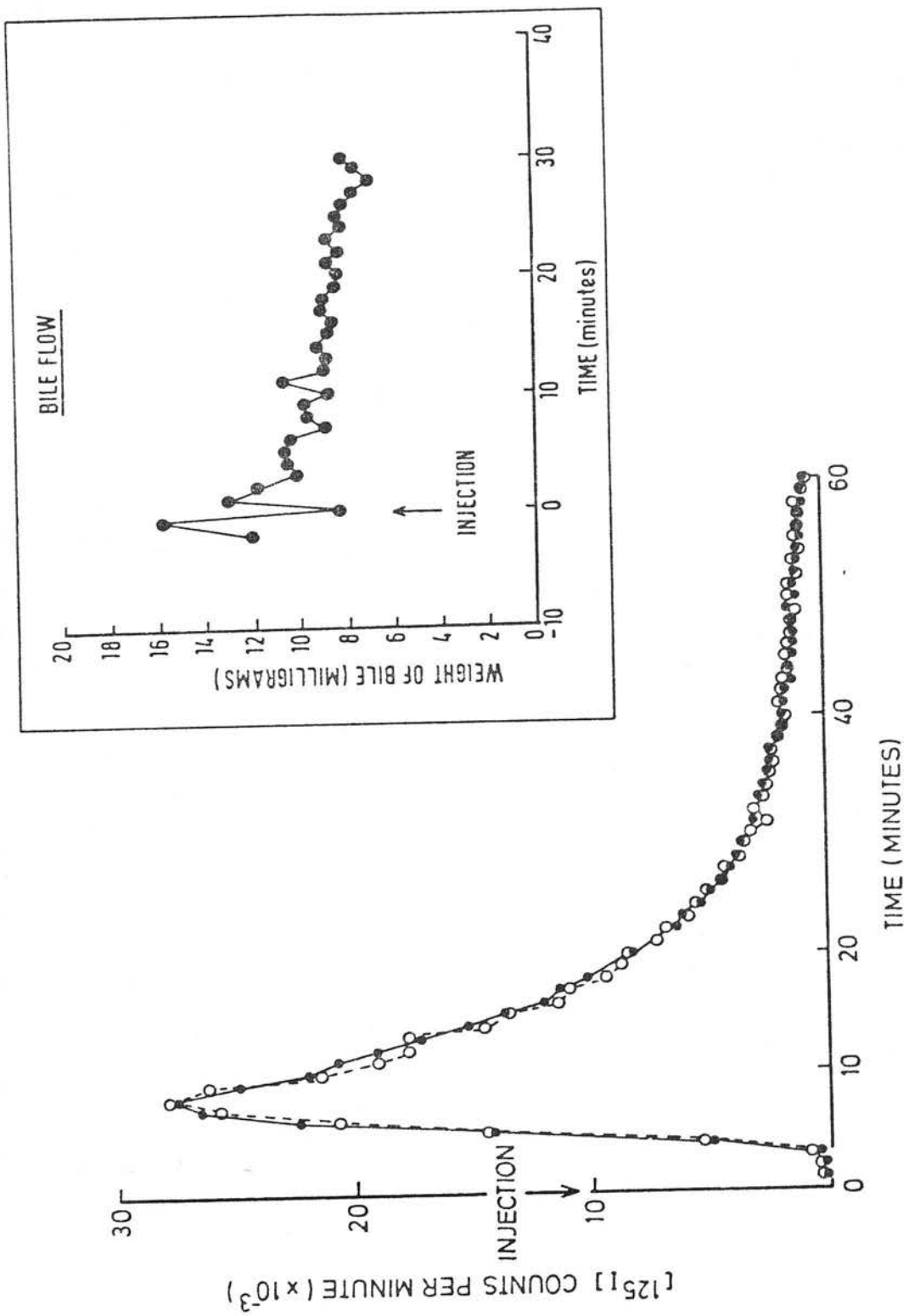

BILE FLOW
WEIGHT OF BILE (MILLIGRAMS)
TIME (minutes)
INJECTION
[125I] COUNTS PER MINUTE (x 10-3)
TIME (MINUTES)
INJECTION

FIGURE 3.11 Hepatic handling of [^{125}I]-3βazidocholylhistamine. Biliary excretion of [^{125}I]-3βazidocholylhistamine in each of two rats after injection (250 fmol, 0.5 μCi) in 154mM saline (75 μl) with 15% ethanol (v/v), into the superior mesenteric vein.

Rat 1 ●——●; Rat 2 o---o; inset shows bile flow over first 30 min following injection.

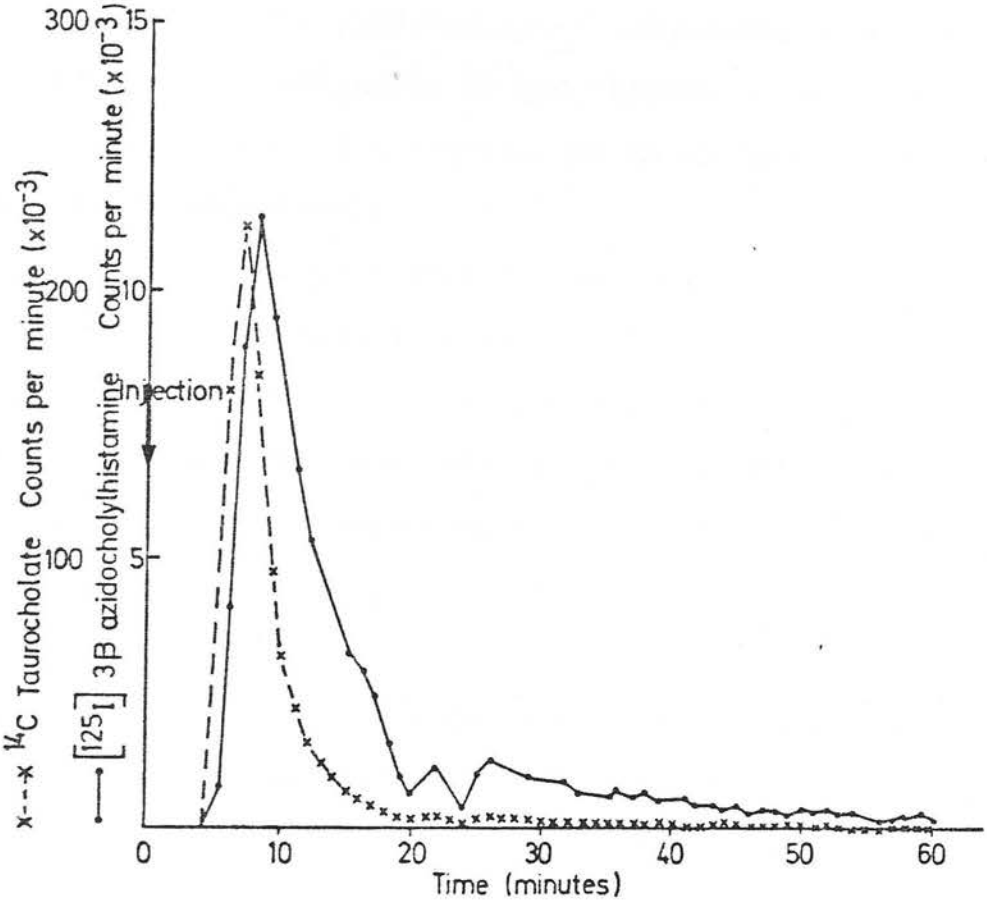


FIGURE 3.12 Hepatic handling of [^{125}I]-3βazidocholylhistamine and [^{14}C]-taurocholate.

Biliary excretion of [^{125}I]-3βazidocholylhistamine and [^{14}C]-taurocholate after injection (125 fmol, 0.25 μCi and 0.8 mmol, 0.5 μCi respectively) in 154 mM saline (75 μl) with 15% ethanol (v/v) into the superior mesenteric vein.

Binding of radiolabelled bile acids to rat hepatic cytosol

The binding of both [^{14}C]-lithocholic acid and [^{125}I]-3βazidocholylhistamine to components of rat hepatic cytosol was investigated by gel filtration on Sephadex G-75 superfine. The results are shown in Figures 3.13 and 3.14 respectively.

[^{14}C]-lithocholic acid showed three major peaks of binding to rat hepatic cytosol. One was a high molecular mass peak of approximately 70 000 Da, one the glutathione S-transferase (Y) peak, and the third a peak of approximately 35 000 Da molecular mass, termed by Sugiyama *et al.* (1983) as Y'. The greatest amount of binding was to the Y peak.

In contrast, [^{125}I]-3βazidocholylhistamine showed one major peak of (non-covalent) binding, the glutathione S-transferase fraction, and several smaller peaks - one was void volume material, molecular mass approximately 100 000 Da or greater, one of the peaks at approximately 70 000 Da, and lastly the Y' peak. The latter two binding peaks can be seen in Figure 3.14.

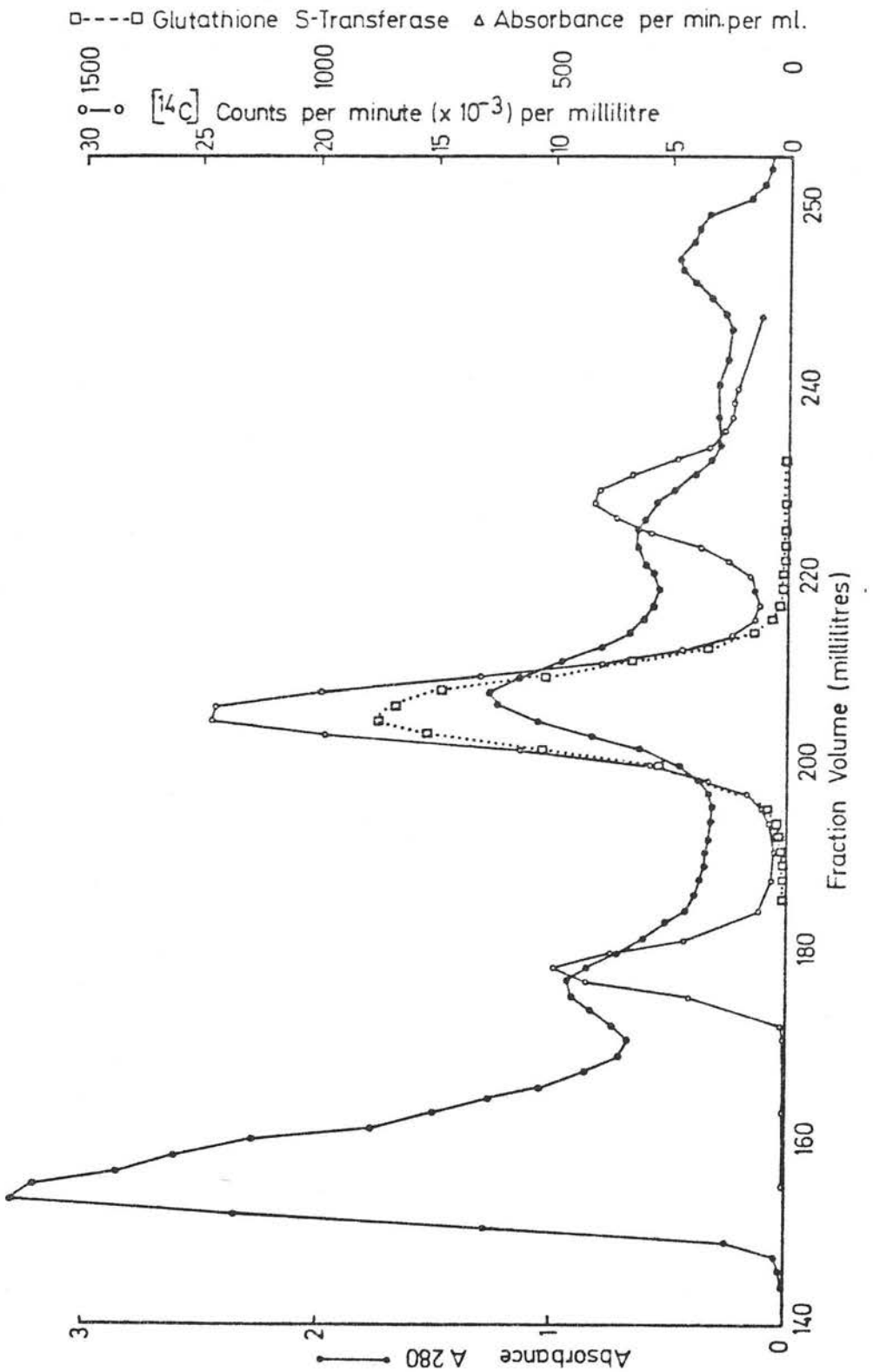

□----□ Glutathione S-Transferase ▵ Absorbance per min.per ml.
[14C] Counts per minute (x 10⁻³) per millilitre
Absorbance A 280
Fraction Volume (millilitres)

FIGURE 3.13 Binding of [^{14}C]-lithocholic acid to rat hepatic cytosol. A portion (100 mg, 5ml) of freshly prepared rat hepatic cytosol was incubated with (carboxy-^{14}C)-lithocholic acid (1.5 μCi) at 4°C for 30 min. The sample was then applied to a column of Sephadex G-75 superfine (2.6 cm x 96 cm) and eluted with buffer A. Flow rate was 9 ml/h , and the fraction volume 1.5 ml.

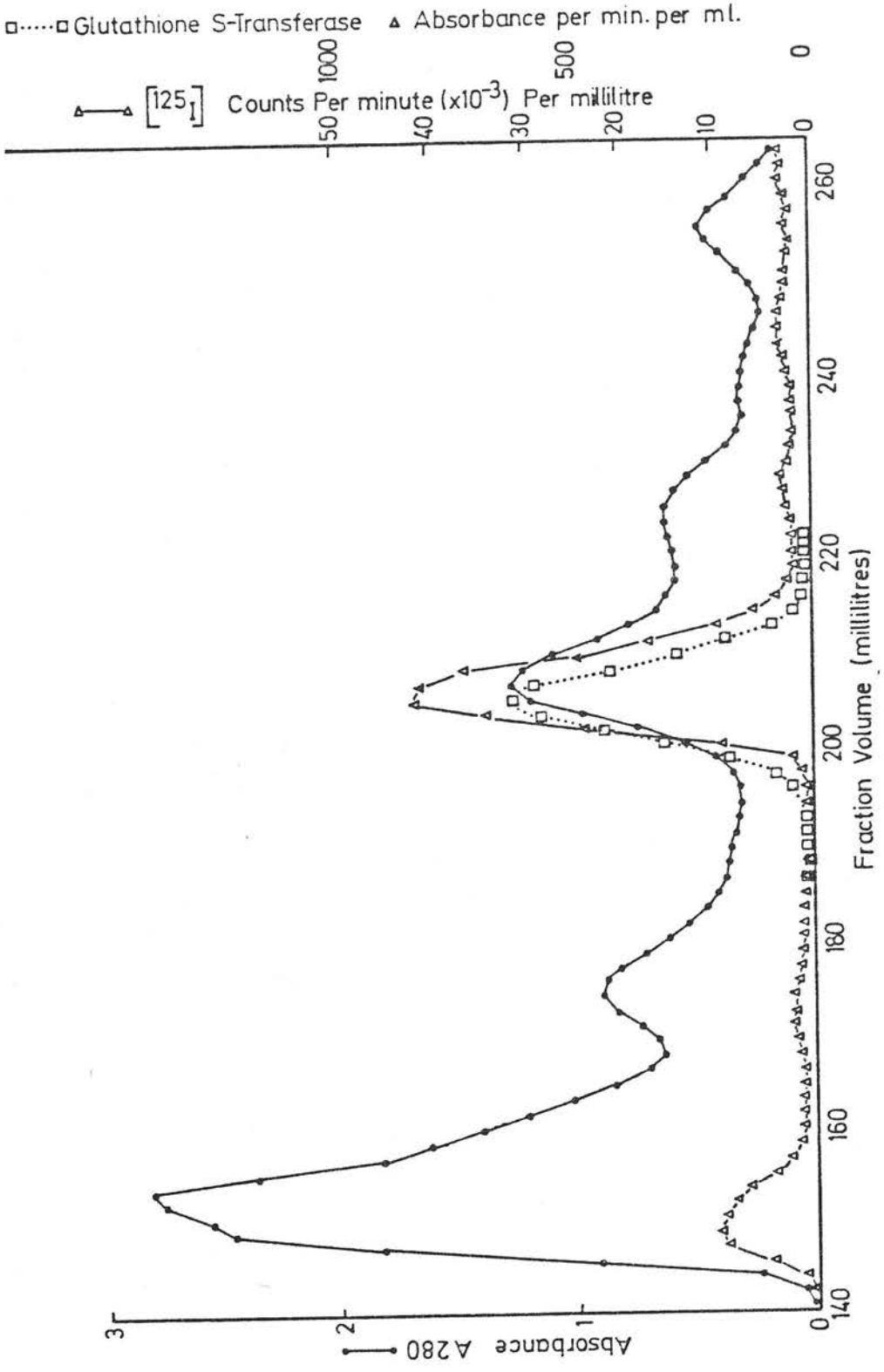

□·····□ Glutathione S-Transferase
▲ Absorbance per min. per ml.
▲——▲ [125I] Counts Per minute (x10-3) Per millilitre
Absorbance A280
Fraction Volume (millilitres)

FIGURE 3.14 Binding of [^{125}I]-3βazidocholylhistamine to rat hepatic cytosol. A portion (100 mg, 5 ml) of freshly prepared rat hepatic cytosol was incubated with [^{125}I]-3βazido--cholylhistamine (5 μCi, 2.5 pmol) at 4°C for 30 min in the dark. The sample was then applied to a column of Sephadex G-75 superfine (2.6 cm x 96 cm) and eluted with buffer A. Flow rate was 9 ml/h and the fraction volume 1.5 ml.

PHOTOAFFINITY LABELLING

Photolysis of [^{125}I]-3βazidocholylhistamine

Before being able to use the molecule as a photoaffinity label, it was necessary to investigate the interaction with ultraviolet light of different wavelengths. Thus, [^{125}I]-3βazidocholylhistamine alone was photolysed in buffer A, and the results are shown in Figure 3.15.

In both cases, with and without the filter, a loss of radioactivity from the parent peak on TLC (Rf 0.79) resulted from the photolysis. At the same time, radioactivity on the TLC plate increased at the origin or at a point close to but clearly distinct from the origin (Rf approximately 0.2). With no filter in position, the solution was exposed to ultra-violet light of wavelength less than 300 nm, and loss of radioactivity was rapid from the parent peak, reappearing mostly at the origin, consistent with the mobility of free iodide. However, some 20% of the radioactivity appeared at a position corresponding to that of iodinated histamine (Rf 0.2).

Photolysis with the filter, cutting out ultraviolet light below 300 nm, resulted in a slower loss of radioactivity from the parent peak, all of which reappeared at a position consistent with iodinated histamine (Rf 0.2). After 30 min, approximately 40% of the radioactivity was still at the position of the original bile acid derivative. In this case, no free iodide was detected.

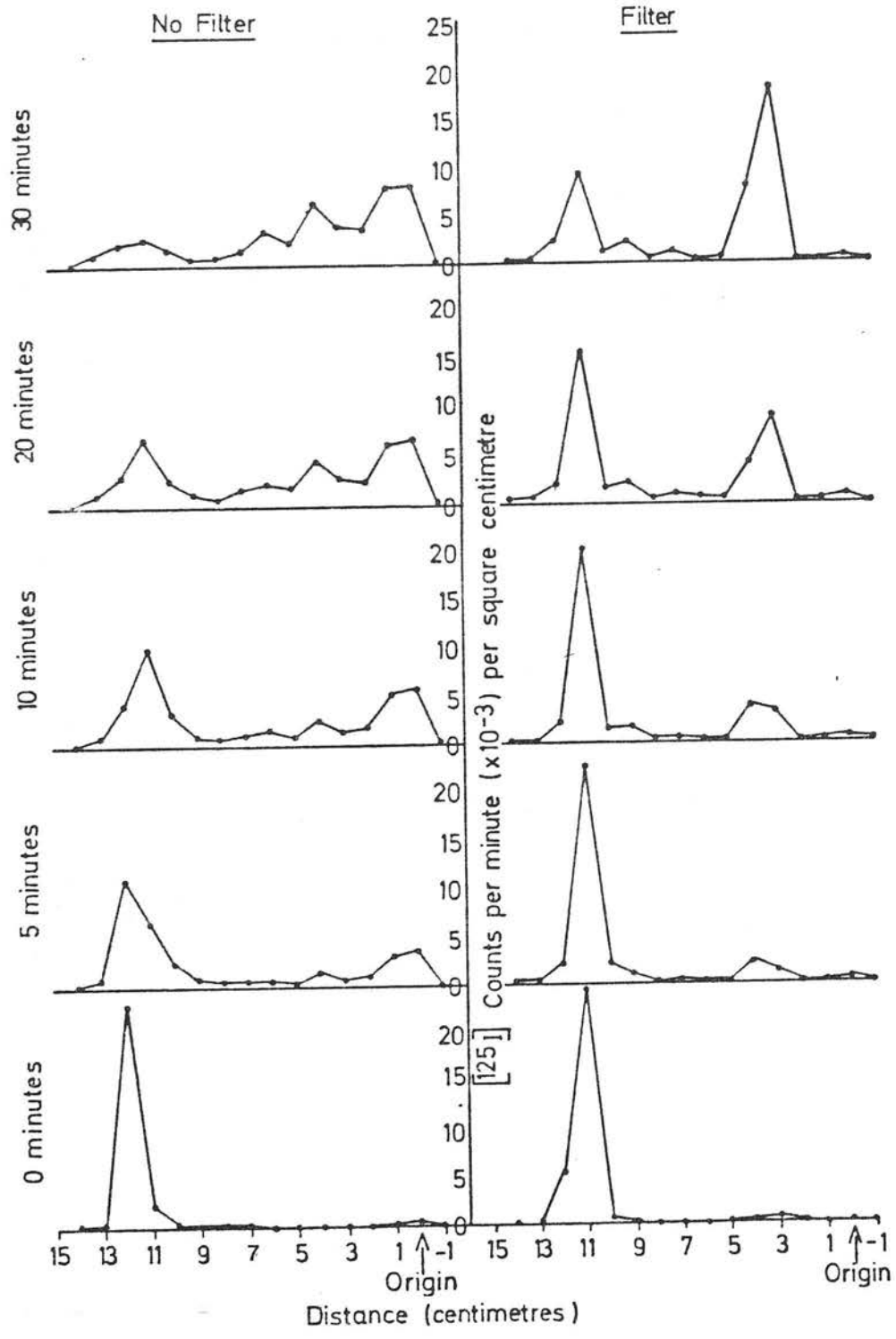

No Filter
Filter
30 minutes
20 minutes
10 minutes
5 minutes
0 minutes
Counts per minute (x10⁻³) per square centimetre
[125 I]
Origin
Origin
Distance (centimetres)

FIGURE 3.15 Photolysis of [^{125}I]-3βazidocholylhistamine. The molecule (5 pmol, 10 μCi) was photolysed, with and without filter, in buffer A (10 ml). Photolysed samples (0,5,10,20 and 30 min) were run on TLC in solvent system 3, scraped and counted for [^{125}I].

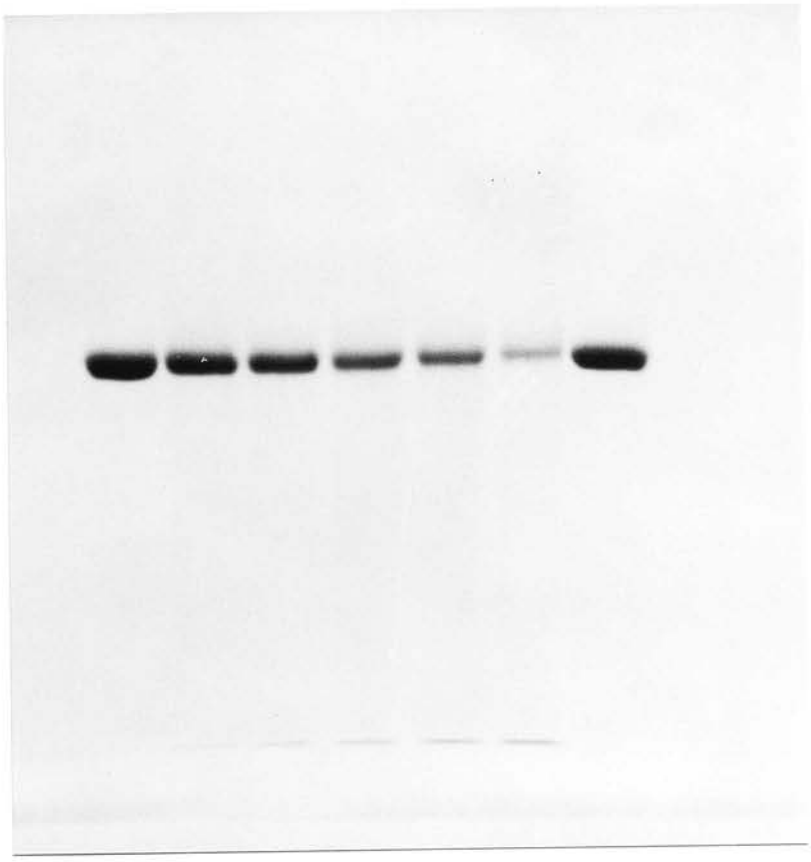
Stability of protein during photolysis

A known bile acid-binding protein, bovine serum albumin, was used in an experiment to determine the effect of ultra-violet light on proteins during photolysis. BSA was photolysed alone, with and without the filter, for different lengths of time, and the results of the SDS/polyacrylamide-gel electrophoresis analysis are shown in Figure 3.16. These results demonstrate that light below 300 nm is the more destructive, with less than 10% of the BSA containing the correct electrophoretic mobility after 60 min photolysis. With the filter in place, however, more than 90% of the BSA is unaltered after the same length of photolysis.

Photoaffinity labelling of bovine serum albumin with [^{125}I]-3βazidocholylhistamine

Bile acid-free BSA was photolysed for different times with the putative photoaffinity label, both with and without the borosilicate glass filter; portions were removed at timed intervals and analysed on SDS/PAGE. The results are shown in Figure 3.17. The extent of covalent labelling of BSA is clearly greater with light above 300 nm than below. Indeed, whereas the amount of radioactivity associated with the BSA band on SDS/PAGE with no filter decreases after 60 min, when the filter is used the extent of labelling of BSA increases until the end of the experiment.

A



TRACK: 1 2 3 4 5 6 7

B

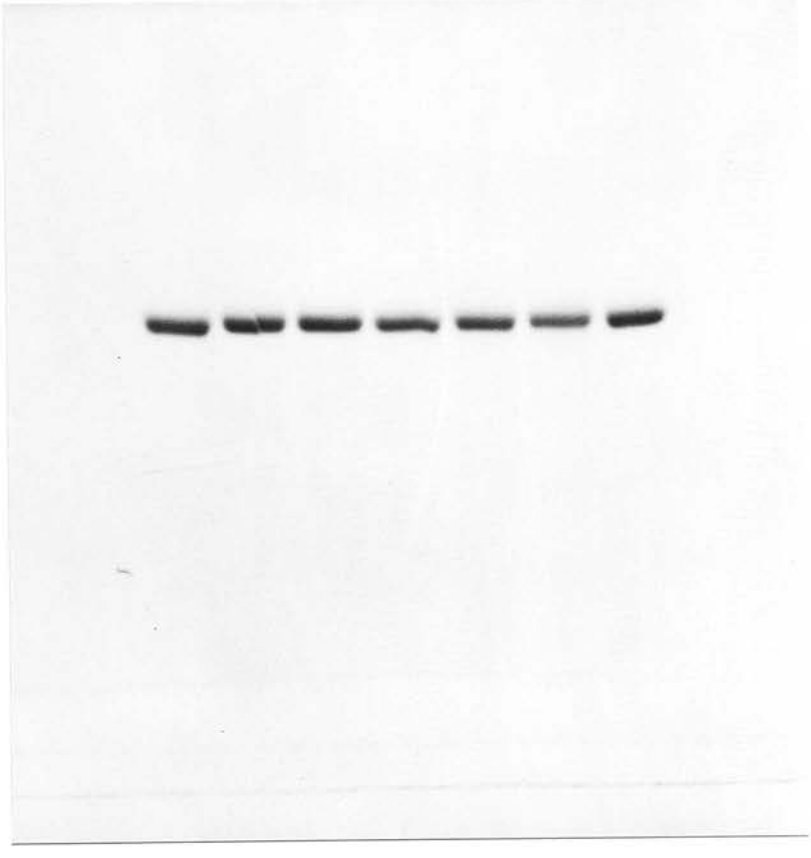


FIGURE 3.16 Stability of bovine serum albumin during photolysis. Stripped BSA (100 mg) in buffer A (10 ml) was photolysed for 60 min without (A) and with (B) borosilicate glass filter.

Tracks 1 and 7 - control (unphotolysed BSA),

Track 2 - 6 - BSA photolysed for 5,10,20,30 and 60 min respectively.

Portions (50 µl) were removed at each interval, prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel.

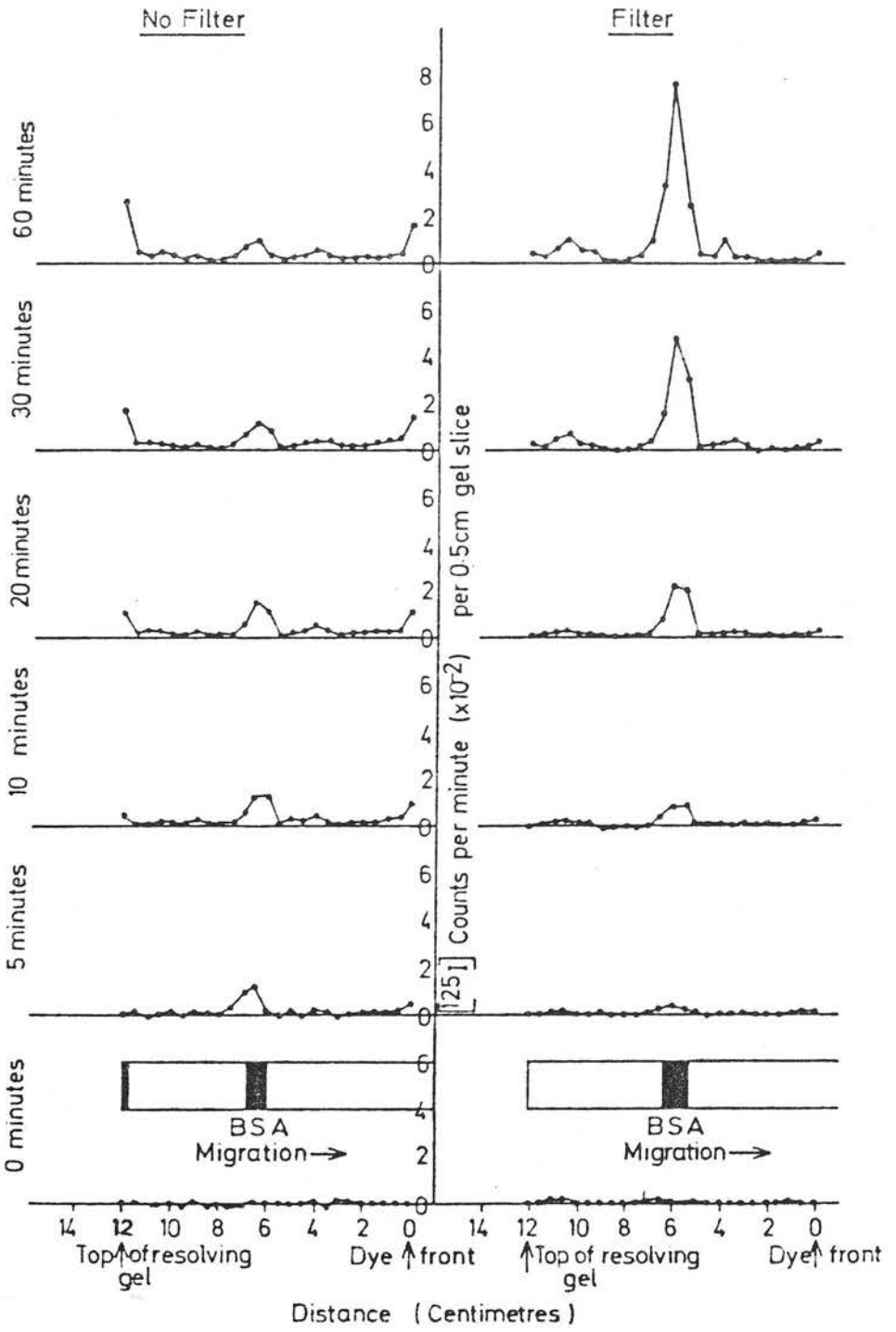

No Filter
Filter
60 minutes
30 minutes
20 minutes
10 minutes
5 minutes
0 minutes
Counts per minute (x10⁻²) per 0.5cm gel slice
[125I]
BSA Migration→
BSA Migration→
14 12 10 8 6 4 2 0
Top of resolving gel
Dye front
Distance (Centimetres)

FIGURE 3.17 Photoaffinity labelling of bile acid-free BSA with [^{125}I]-3βazidocholylhistamine. Stripped BSA (100 mg) in buffer A (10 ml) was photolysed in the presence of [^{125}I]-3β azidocholylhistamine (5 pmol, 10 μCi) both with and without the borosilicate glass filter, for 60 min. At 0,5,10,20,30 and 60 min, portions (50 μl) were removed, prepared for SDS/polyacrylamide-gel electrophoresis, and analysed on a 12% resolving gel.

Inset at bottom shows position of BSA band on gel.

As an alternative means of demonstrating covalent attachment of the label to protein, the photolysed solutions (after 60 min) were applied to columns of Sephadex G-100 fine and eluted with buffer A (Figure 3.18). A control solution, (Figure 3.18c) where no photolysis had taken place, shows the BSA elutes at a volume of approximately 100 ml, with no associated radioactivity. The label appears in the salt volume (250 ml) of the column.

Analysis of the Sephadex G-100 eluate obtained from the photolysed mixture, shown in Figure 3.18 A and B, revealed the free unreacted label again eluted at 200 - 250 ml in the salt volume of the column, but there was also radioactivity associated with the BSA. When the filter was used during photolysis (B), the BSA/radioactivity eluted at approximately the same volume as the BSA in the control (C) i.e., 100 ml. In contrast, with no filter used (A), the majority of the BSA/radio-activity appeared in the void volume of the column (approximately 50 ml), with only a small amount of BSA alone at a volume of 100 ml. The void volume material represents BSA aggregates formed during photolysis, and is consistent with the high molecular mass band seen at the top of the resolving gel in Figure 3.17, with radioactivity associated.

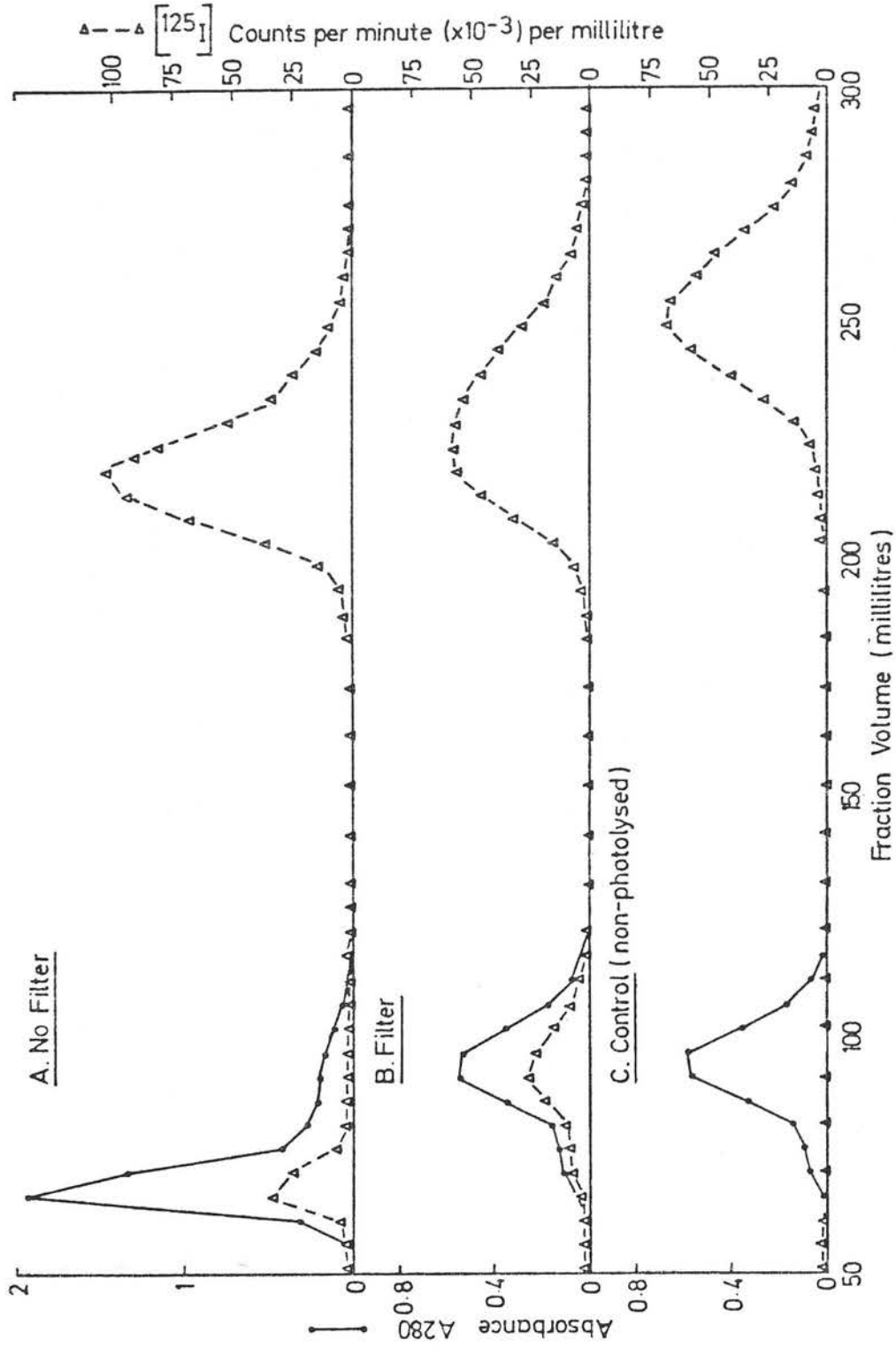

Δ— —Δ [125I] Counts per minute (x10⁻³) per millilitre
A. No Filter
B. Filter
C. Control (non-photolysed)
Absorbance A280
Fraction Volume (millilitres)

FIGURE 3.18 Gel filtration of photolysis products.

After 60 min photolysis without (A) and with (B) the borosilicate glass filter, the solutions (10 ml) of BSA and [^{125}I]-3βazidocholylhistamine (10 mg/ml and 0.5 pmol/ml respectively) were applied to columns of Sephadex G-100 fine (2.6 cm x 42 cm) and eluted with buffer A. Flow rate was 21 ml/h and the fraction volume 5 ml.

A control (C), where no photolysis had taken place, is also shown.

Photoaffinity labelling of rat material

(i) Whole rat hepatic cytosol

A portion of rat hepatic cytosol was photolysed in the presence of [^{125}I]-3βazidocholylhistamine for a total period of 3 h and samples were taken at intervals for analysis by SDS/polyacrylamide-gel electrophoresis (Figure 3.19). A portion of the final reaction mixture was analysed by gel filtration chromatography on Sephadex G-75 superfine (Figure 3.20).

The SDS/polyacrylamide-gel electrophoresis showed the appearance of labelling after 1 h, increasing to 2 h, and then 3 h. The labelling is associated with protein bands of approximate molecular masses 60 000 Da (albumin), 45 000 Da (hydroxycholanoyl transferase), 35 000 Da and 25 000 Da (glutathione S-transferase). The labelling at 35 000 Da is suggested to be the Y' fraction, containing new binding protein*. This overall picture is supported by gel filtration of the 3 h photolysed sample, which shows labelling of the Y' region. Radioactivity can also be seen at the top of the resolving gel, still increasing after 3 h. This is thought to be due to photolytically-mediated aggregations of labelled proteins, forming so large a mass as to be unable to enter the resolving gel.

*NOTE: As the cytosol used in these experiments was derived from unperfused liver, consideration must be given to the possibility of the contribution of plasma proteins to these results.

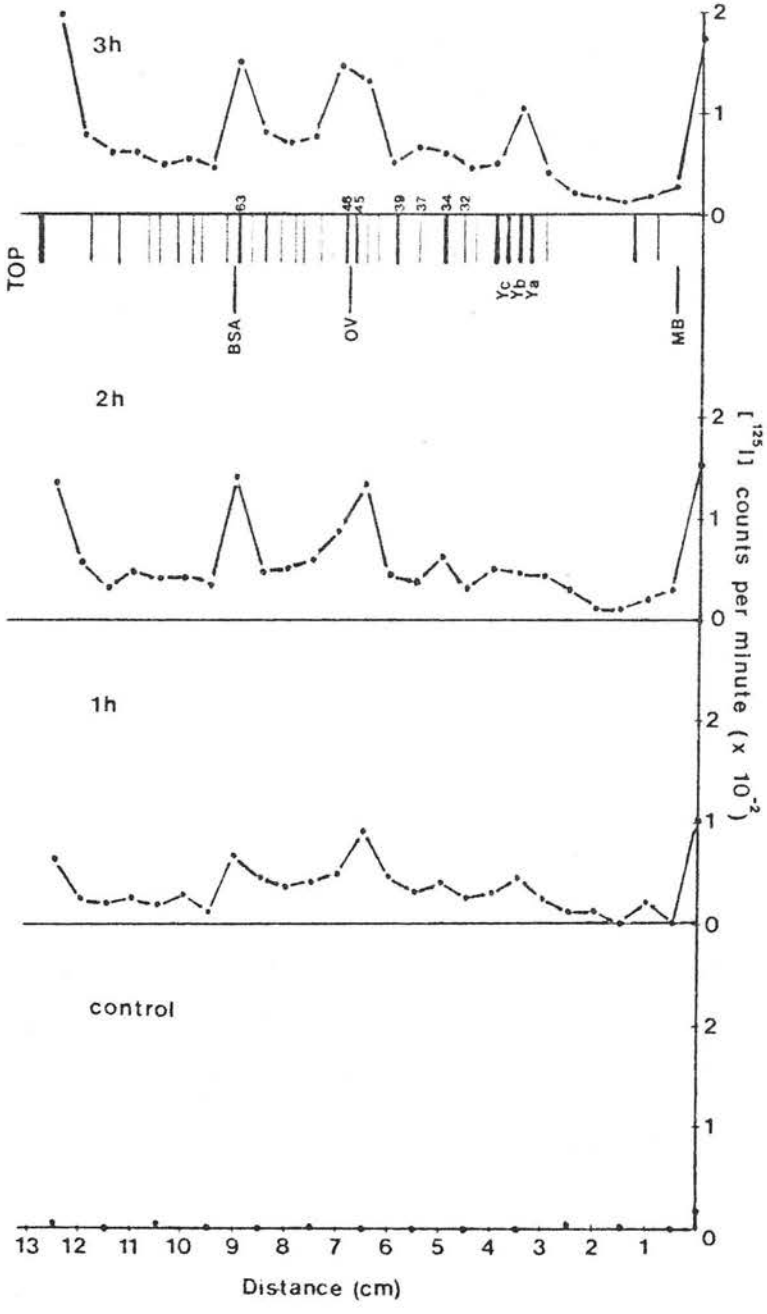

3h
2h
1h
control
TOP
63
48
45
39
37
34
32
BSA
OV
Yc
Yb
Ya
MB
2
1
0
[125I] counts per minute (x 10-2)
13 12 11 10 9 8 7 6 5 4 3 2 1
Distance (cm)

FIGURE 3.19 Photoaffinity labelling of whole rat hepatic cytosol with [^{125}I]-3βazidocholylhistamine.

A portion of rat hepatic cytosol (100 mg, 10 ml) was photolysed for a total of 3 h with [^{125}I]-3βazidocholylhistamine (10 μCi, 5 pmol). Samples were taken at 0, 1 h, 2 h and 3 h, prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% gel. Three proteins were used as standards - BSA (66 000 Da), ovalbumin (45 000 Da) and myoglobin (17 000 Da). The gel tracks were cut into 0.5 cm slices and counted for ^{125}I.

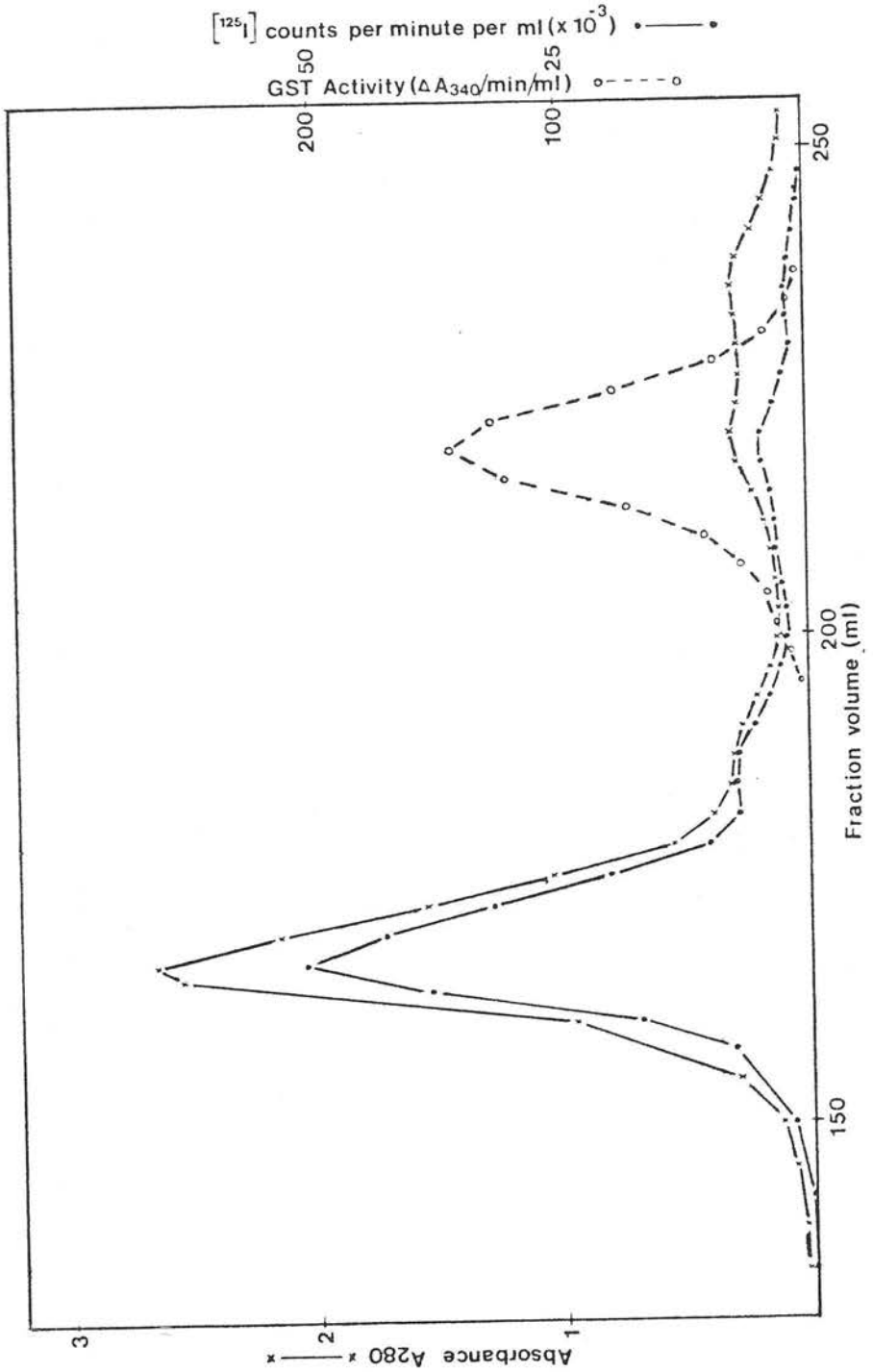

[125I] counts per minute per ml (x 10-3)
GST Activity (ΔA340/min/ml)
Absorbance A280
Fraction volume (ml)
50
25
200
100
3
2
1
150
200
250

FIGURE 3.20 Gel filtration of photoaffinity labelled rat hepatic cytosol on Sephadex G-75 superfine.

A portion of rat hepatic cytosol (50 mg, 5 ml) which had been photolysed with [^{125}I]-3βazidocholylhistamine (10 μCi, 5 pmol) for 3 h, was applied to a column (2.6 cm x 100 cm) of Sephadex G-75 superfine and eluted with buffer A. Flow rate was 9 ml/h and the fraction volume 1.5 ml.

(ii) Glutathione S-transferase fraction (Y peak)

A portion of rat liver cytosol from the second Sephadex G-75 superfine step of the Y′ protein purification scheme (see next section), containing mainly the glutathione S-transferases, was photolysed for a total period of 2 h. Samples were taken at intervals and analysed by SDS/polyacrylamide-gel electrophoresis - the results are shown in Figure 3.21.

The majority of labelling on the gel is associated with the glutathione S-transferase subunits Ya, Yb and Yc. However, a smaller degree of labelling is seen with proteins in the 30 000 -40 000 Da range and with two proteins of approximate molecular mass 15 000 Da. The extent of labelling still apparently increases after 2 h, although the amount of radioactivity found at the top of the resolving gel (Figure 3.21, left) also increases. This may again be due to photolytically-mediated aggregation of labelled proteins, as explained in the previous section.

(i.e all components of the Y′ fraction on sephadex).

Purified glutathione S-transferases were also photoaffinity labelled and assessed for the covalent incorporation of [^{125}I]-3β-azidocholylhistamine. The results of this experiment, using glutathione S-transferases AA, A, D and F, are shown in Table 3.2.

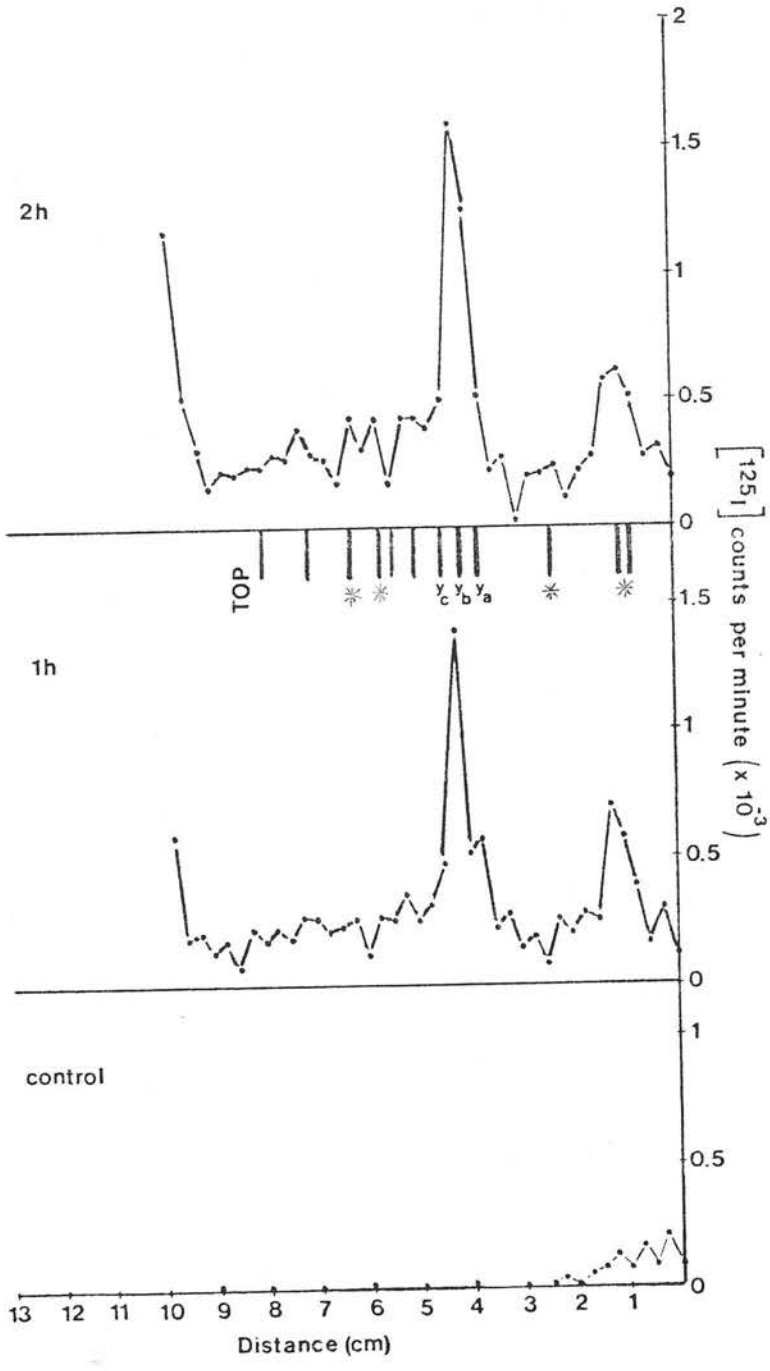

2h
1h
control
TOP
y_c y_b y_a
2
1.5
1
0.5
0
1.5
1
0.5
0
1
0.5
0
13 12 11 10 9 8 7 6 5 4 3 2 1
Distance (cm)
[125I] counts per minute (x 10-3)

FIGURE 3.21 Photoaffinity labelling of glutathione S-transferase fraction with [^{125}I]-3βazidocholylhistamine.

A portion of the glutathione S-transferase fraction (30 mg, 10 ml) from rat hepatic cytosol was photolysed for a total of 2 h with [^{125}I]-3β azidocholylhistamine (10 μCi, 5 pmol). Samples were taken at 0, 1 h and 2 h, prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel. The gel tracks were cut into 0.5 cm slices and counted for [^{125}I].

These protein bands as marked * on the gel track reproduced opposite are components of the Y' fraction. The three subunits of the glutathione S-transferases are indicated as shown (Ya, Yb, Yc).

Table 3.2. Photoaffinity labelling of purified glutathione S-transferases

Glutathione S-transferase	Subunit composition	Incorporation of [^{125}I] per 50 µg (CPM x 10^{-3})
AA	YcYc	160
A	Yb_1Yb_1	119
D	Yb_2Yb_2	267
F	YaYa	186

Portions (50 µg) of glutathione S-transferases AA, A, D and F were photolysed with [^{125}I]-3β-azidocholylhistamine (2.5 µCi, 1.25 pmol) for 15 min. After TCA precipitation and extensive washing, the proteins were taken up in buffer A, containing 1% SDS (w/v) (500 µl). Samples were applied to a column (1.5 cm x 20 cm) of Sephadex G-25 fine and eluted with the same buffer. Flow rate was 25 ml/h and fraction volume 4 ml.

PURIFICATION OF Y' PROTEINS FROM RAT HEPATIC CYTOSOL

Y' Protein Purification

The purification scheme for Y' proteins is shown in Table 2.4, and essentially consists of three gel filtration steps, followed by ion exchange, chromatofocusing and finally hydroxyapatite chromatography.

Cytosol (approximately 7 g of protein) from 90-100 g of rat liver was applied to a column of Sephadex G-100 and eluted with buffer A (Figure 3.22). The glutathione S-transferase fractions were pooled and concentrated by ultrafiltration.

The concentrated Y peak (approximately 2 g of protein, 80 ml) was centrifuged (20 min. 4°C, 20 000 g) and applied to a column of Sephadex G-75 superfine which was eluted with buffer A. The resulting elution profile is shown in Figure 3.23. From the results of SDS/polyacrylamide-gel electrophoresis, those fractions which contained polypeptides in the range 30 000 Da to 15 000 Da were pooled (Figure 3.24). As Figure 3.24 shows, the Y' proteins were not completely resolved from the glutathione S-transferases. A second gel filtration step on Sephadex G-75 superfine was generally required to achieve this separation.

The partially purified Y' peak (approximately 40 ml, 500 mg of protein) was re-applied to the column of Sephadex G-75 superfine and eluted with buffer A as

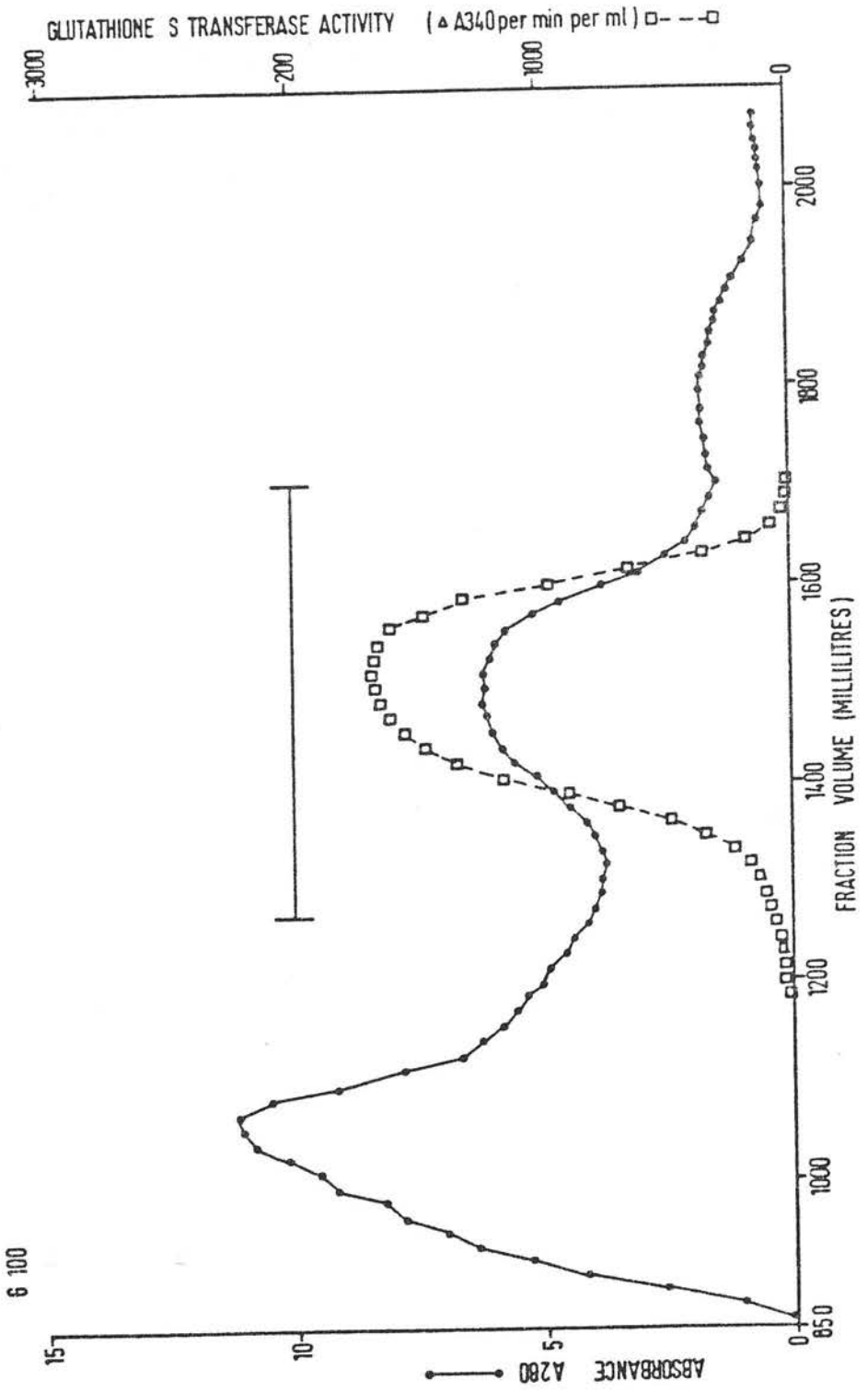

GLUTATHIONE S TRANSFERASE ACTIVITY (Δ A340 per min per ml) □- - -□
3000
2000
1000
0
2000
1800
1600
1400
1200
1000
850
FRACTION VOLUME (MILLILITRES)
ABSORBANCE A280
15
10
5
0
6 100

FIGURE 3.22 Gel filtration of rat hepatic cytosol on Sephadex G-100. Freshly prepared rat hepatic cytosol (approximately 7 g of protein) was applied to two columns in series of Sephadex G-100 fine (total volume 2675 ml) and eluted with buffer A. Flow rate was 22.5 ml/h and the fraction volume 3.7 ml. The bar in the figure represents the material which was pooled for the next stage of the purification.

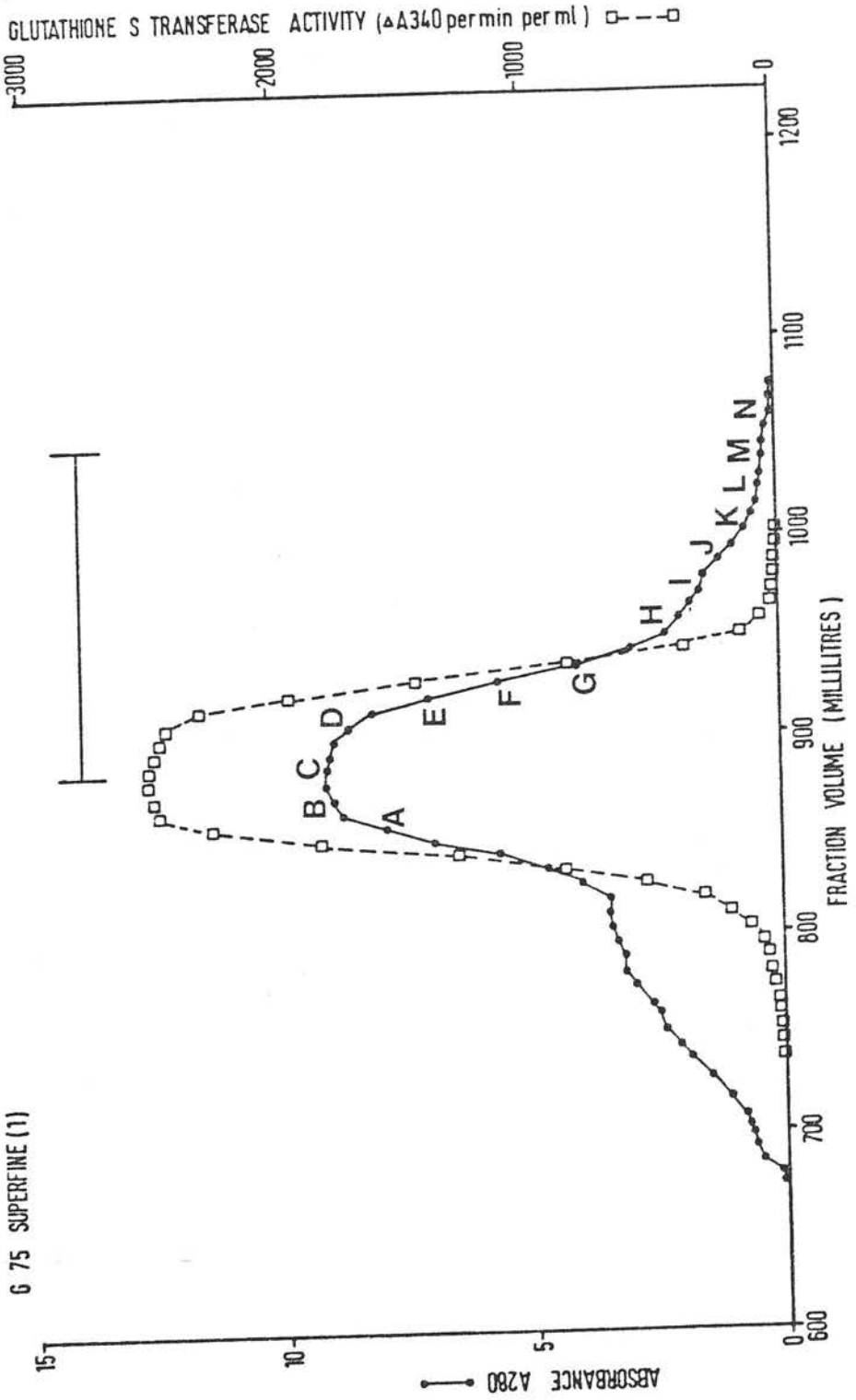

GLUTATHIONE S TRANSFERASE ACTIVITY (ΔA340 per min per ml)
3000
2000
1000
0
1200
1100
1000
900
800
700
600
FRACTION VOLUME (MILLILITRES)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
G 75 SUPERFINE (1)
15
10
5
0
ABSORBANCE A280

FIGURE 3.23. Gel filtration of the Y′ peak on Sephadex G-75 superfine.

The glutathione S-transferase peak from the G-100 step was applied to two columns in series (total volume 2050 ml) of Sephadex G-75 superfine and eluted with buffer A. Flow rate was 15 ml/h and the fraction volume 2.5 ml.

The bar in the figure represents the material which was pooled for the next stage of the purification. The letters beside certain fractions indicate those which were analysed by SDS/polyacrylamide-gel electrophoresis (Figure 3.24).

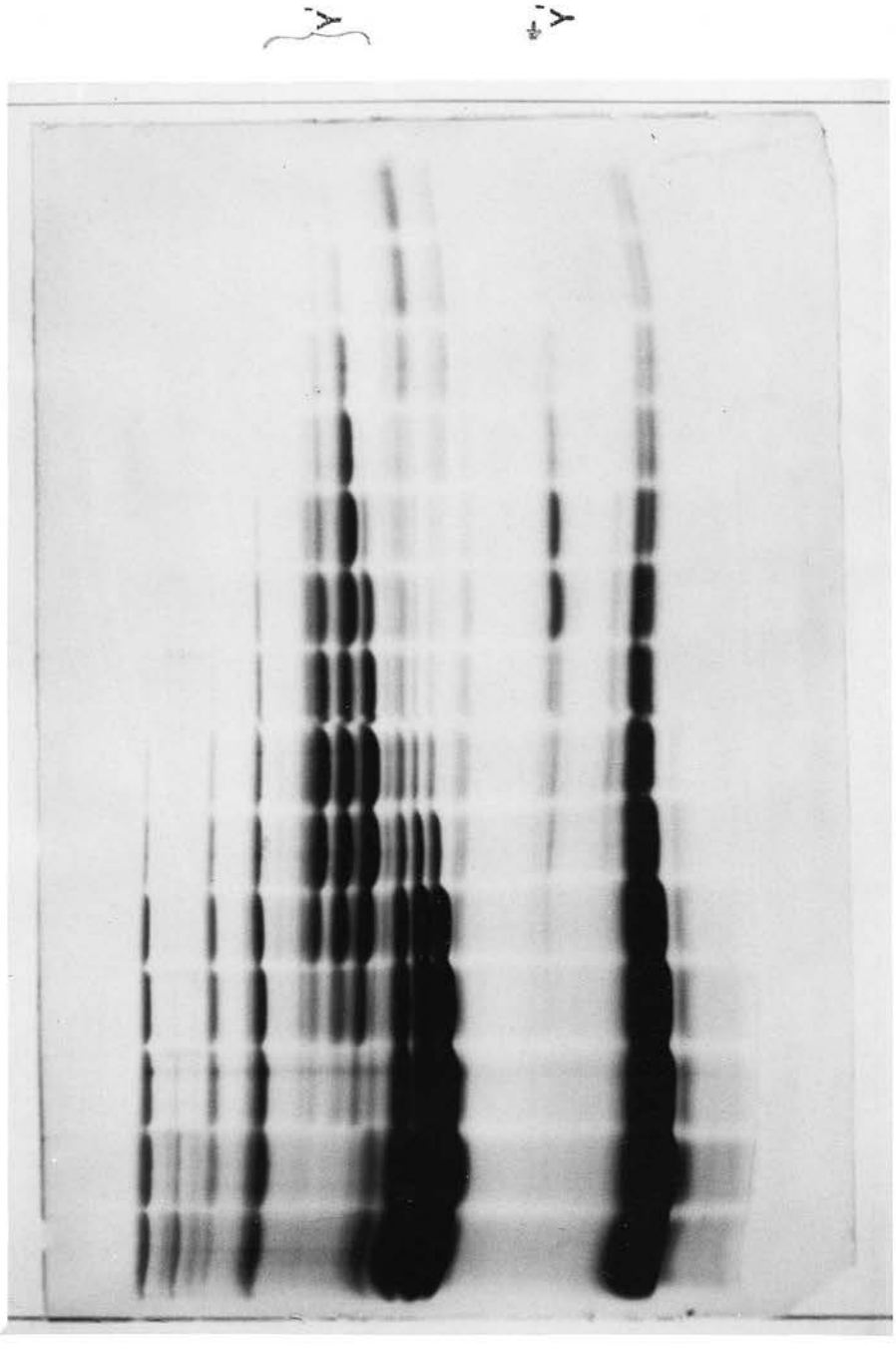

-Y
+Y
FRACTION: A B C D E F G H I J K L M N

FIGURE 3.24 SDS/polyacrylamide-gel electrophoresis of fractions from first Sephadex G-75 superfine step.

Portions of the fractions from the first G-75 gel filtration step, represented by letters in Figure 3.23 and also shown above, were prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel.

described above. Figure 3.25 shows that the Y' peak was almost completely resolved from the Y peak and, on the basis of SDS/polyacrylamide-gel electrophoresis of the peak fractions, (Figure 3.26), the material represented by the bar was pooled for the next purification step.

The pooled Y' peak was applied to a column of DEAE Sephadex and eluted as described in the Materials and Methods. The resulting elution profile is shown in Figure 3.27 and the electrophoretic analysis of the peak fractions in Figure 3.28. A total of 12 peaks were eluted from the ion-exchange column. Each was tested for glutathione S-transferase activity; only the void volume fraction, peak 1, showed any significant enzyme activity (Table 3.3). Bands which co-migrated during electrophoresis with glutathione S-transferase subunits could be seen in peak 1. The molecular masses of the proteins from each peak are summarised in Table 3.4.

Portions of each peak obtained from DEAE-Sephadex were subjected to photoaffinity labelling with [^{125}I]-3βazidocholylhistamine, in order to determine the bile acid-binding potential of the proteins. This is described in more detail later (p.167).

On the basis of the results from photoaffinity labelling, peaks 5, 6, 7 and 8 were pooled, as represented by the bars in Figure 3.27, and dialysed against buffer C. Each peak was then individually applied to chromatofocusing columns of PBE 94 and eluted with buffer D.

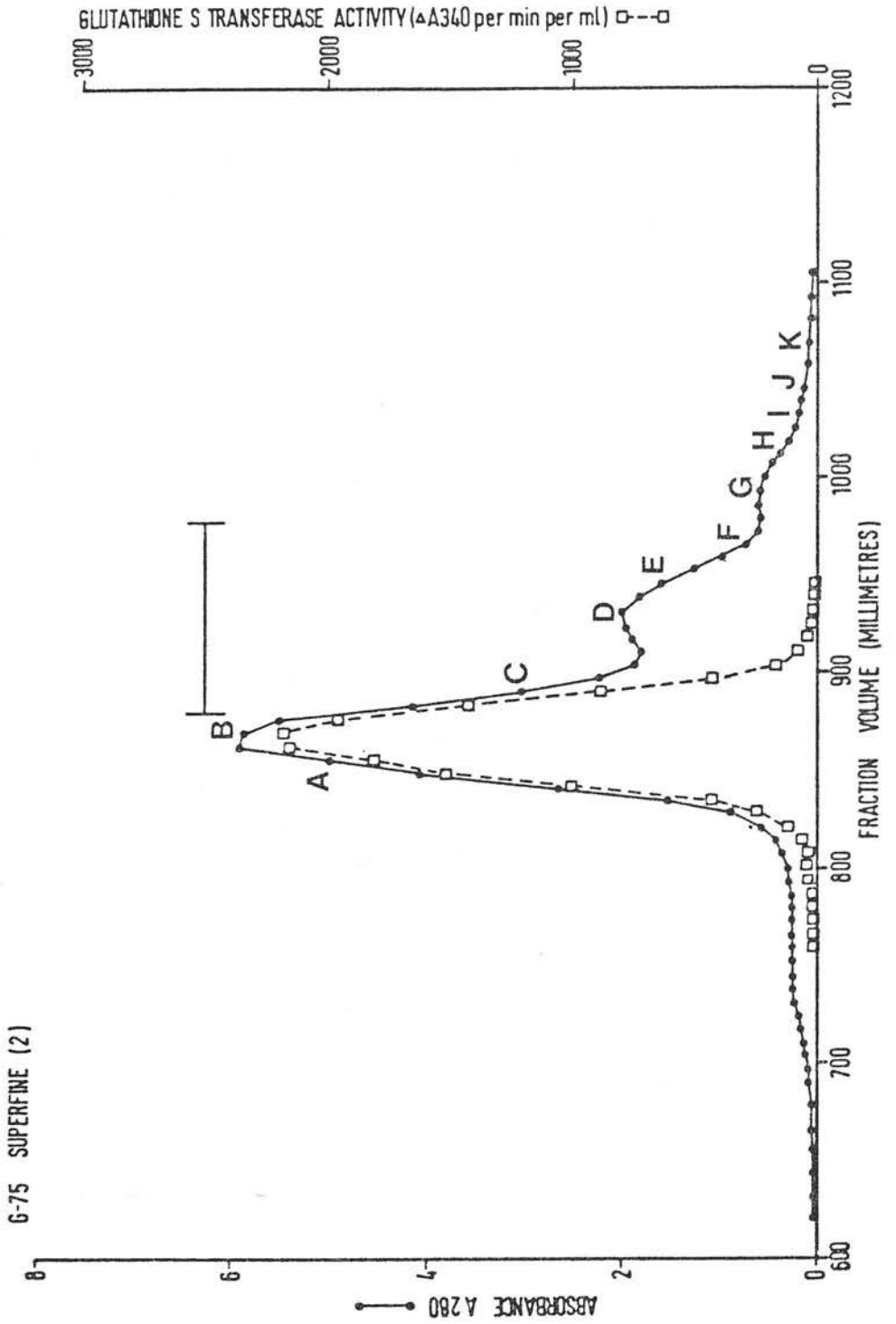

GLUTATHIONE S TRANSFERASE ACTIVITY (ΔA340 per min per ml)
3000
2000
1000
0
1200
1100
1000
900
800
700
600
FRACTION VOLUME (MILLIMETRES)
A
B
C
D
E
F
G
H
I
J
K
ABSORBANCE A 280
8
6
4
2
0
G-75 SUPERFINE (2)

FIGURE 3.25 Gel filtration of partially purified Y′ peak on Sephadex G-75 superfine.

The partially purified Y′ peak from the first G-75 step was re-applied to the two columns of Sephadex G-75 superfine (total volume 2050 ml) and eluted with buffer A. The flow rate was 15 ml/h and the fraction volume 2.5 ml. The bar in the figure represents the material which was pooled for the next stage of the purification. The letters beside certain peak fractions indicate those which were analysed by SDS/polyacrylamide-gel electrophoresis (Figure 3.26).

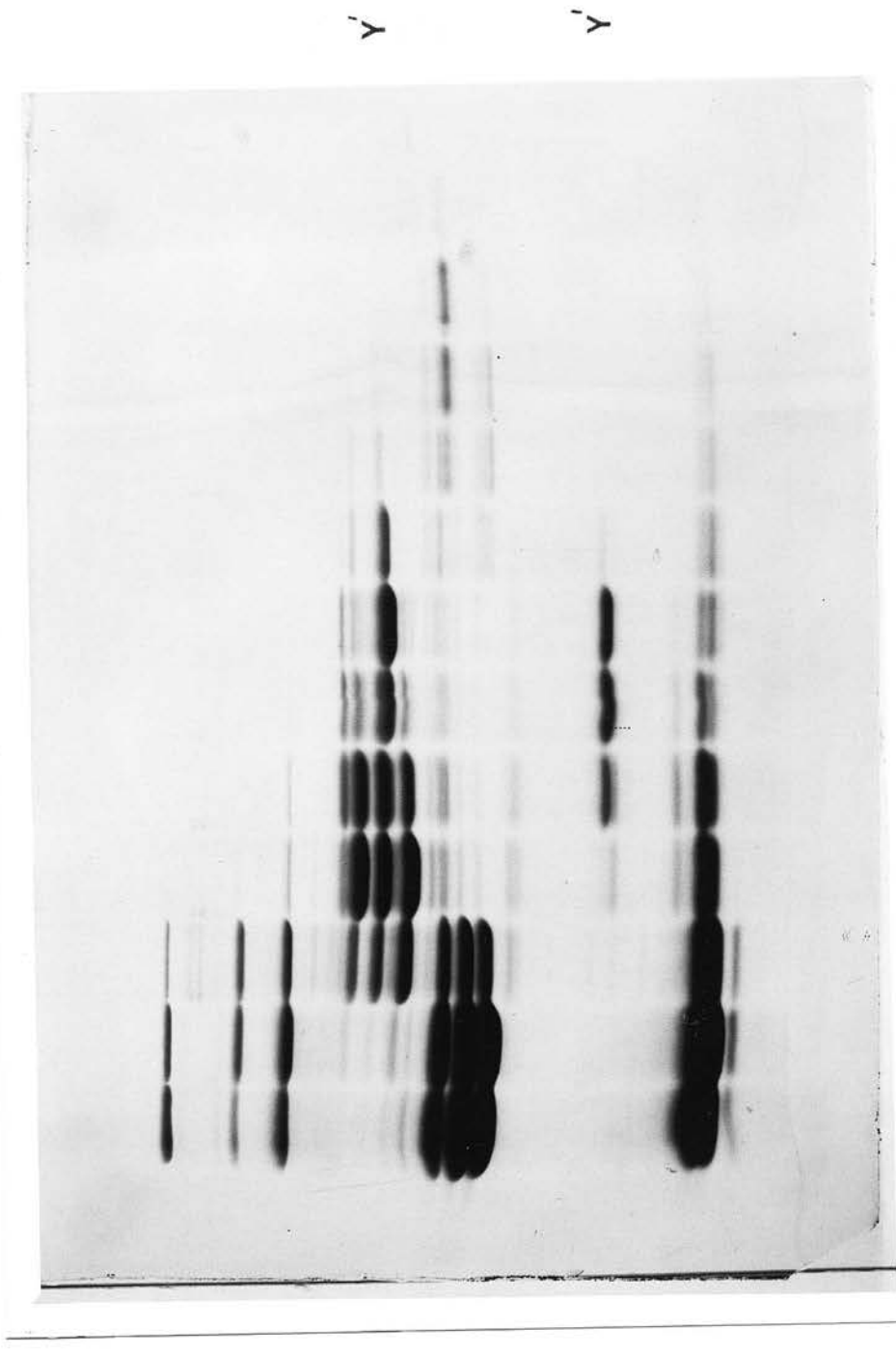

Y'
Y'
FRACTION: A B C D E F G H I J K

FIGURE 3.26 SDS/polyacrylamide-gel electrophoresis of peak fractions from second Sephadex G-75 step.

Portions of the fractions from the second G-75 step, represented by letters in Figure 3.25 and also shown opposite, were prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel.

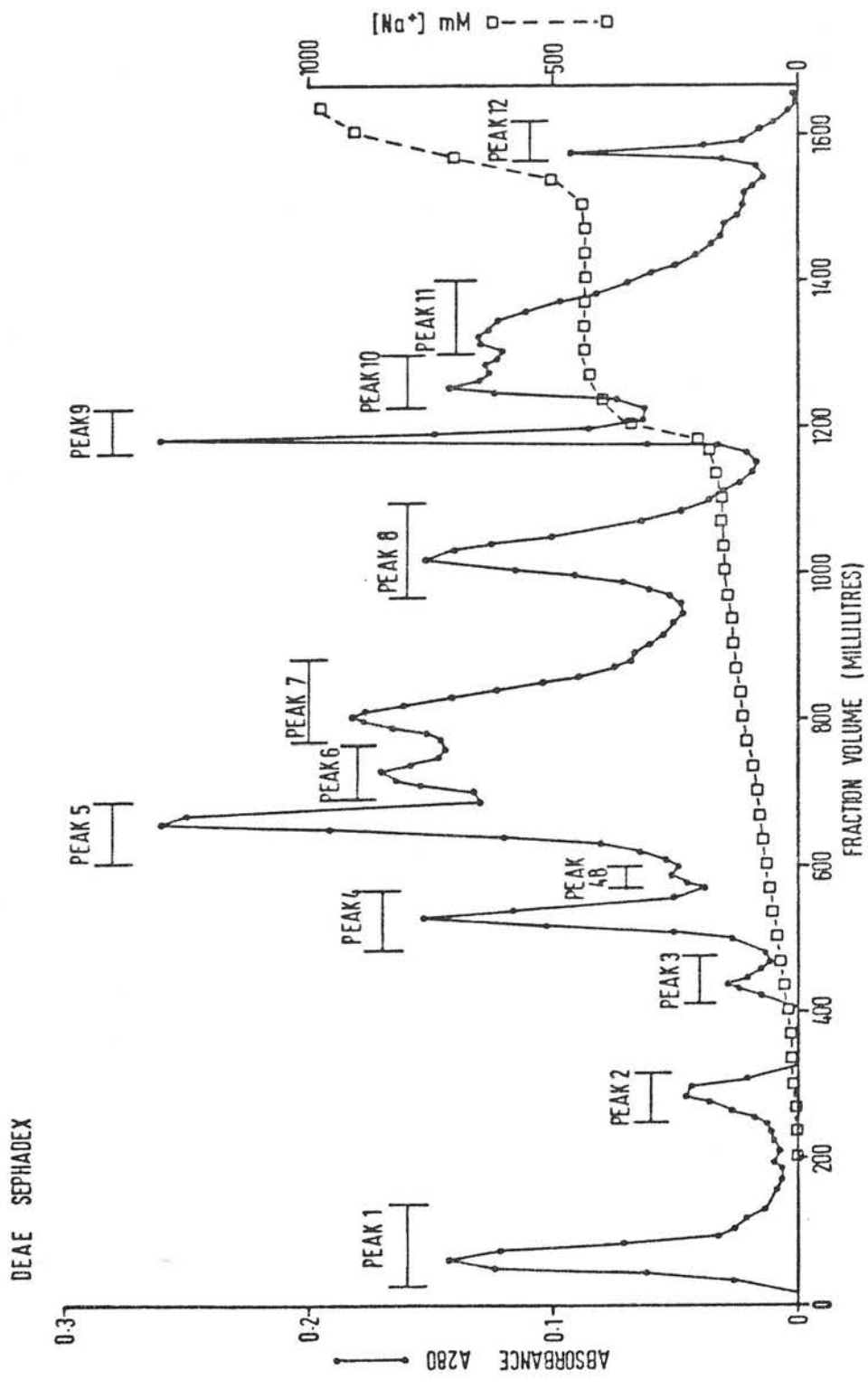

DEAE SEPHADEX
ABSORBANCE A280
0·3
0·2
0·1
0
[Na+] mM
1000
500
0
FRACTION VOLUME (MILLILITRES)
0
200
400
600
800
1000
1200
1400
1600
PEAK 1
PEAK 2
PEAK 3
PEAK 4
PEAK 4B
PEAK 5
PEAK6
PEAK 7
PEAK 8
PEAK9
PEAK10
PEAK 11
PEAK12

FIGURE 3.27 Y′ peak on DEAE Sephadex.

The pooled Y′ peak from the second gel filtration step (approximately 100 ml, 175 mg protein) was applied to a column (2.2 cm x 25 cm) of DEAE Sephadex A-50 (total volume 95 ml) equilibrated with buffer B. Elution was as described in the Material and Methods. Portions of each of the peak fractions were prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel (Figure 3.28).

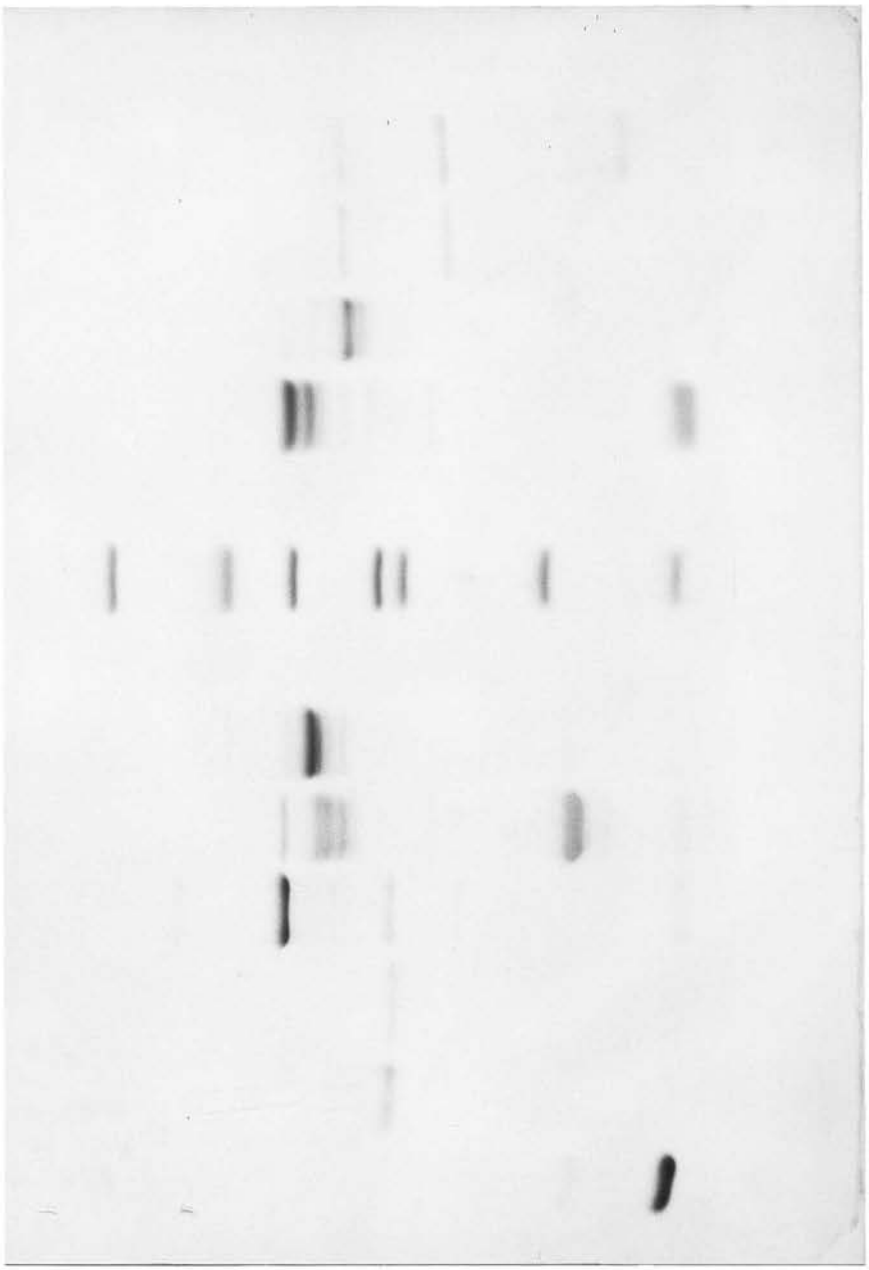

PEAK: 1 2 3 4 5 6 marker 7 8 9 10 11

FIGURE 3.28 SDS/polyacrylamide-gel electrophoresis of Y' proteins from DEAE Sephadex.

Portions of each peak fraction from the DEAE Sephadex step (Figure 3.27) were prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel. The marker protein had seven standards and a typical calibration is shown in Figure 2.1.

Table 3.3: Glutathione S-transferase activity of Y' peaks from DEAE Sephadex

Peak	Glutathione S-transferase activity A_{340}/min/ml
1	21
2	0.3
3	0.6
4	0.6
5	0
6	0
7	0
8	0.1
9	0.1
10	0
11	0
12	0

Table 3.4: Molecular masses of Y' proteins from DEAE Sephadex

Peak	Molecular mass of protein bands (Da)
1	17 700; 14 900;
2	28 600;
3	28 600;
4	50 600; 37 900; 28 600;
5	37 900; 34 400; 33 500; 32 700; 17 400; 16 000; 13 000;
6	35 300; 34 400; 32 400; 17 500;
7	37 200; 35 000; 13 100; 12 700;
8	31 800; 30 400;
9	31 800; 24 000;
10	31 800; 24 000; 15 100;
11	24 000; 15 100;
12	NO VISIBLE BANDS.

Calibration of a 12% resolving gel using a seven protein standard - see Figure 2.1. The approximate molecular masses given here are derived from the SDS/polyacrylamide-gel in Figure 3.25; no attempt has been made here to indicate whether a band is a major or minor component of a peak.

The elution profiles of peaks 5 to 8 are given in Figures 3.29 to 3.32 respectively. Figures 3.33 and 3.34 show the electrophoretic analysis of the peak fractions from each chromatofocusing run. The molecular masses of the protein bands are summarised in Table 3.4.

Each of the peaks from the four chromatofocusing runs were photoaffinity labelled with [^{125}I]-3βazidocholylhistamine to test for bile acid-binding. This is described in more detail later (p.167), and the results given in Table 3.5.

Those proteins which were photoaffinity labelled could be divided into two groups - those with a high specific labelling following photolysis ($\geq$ 1 x 10^5 counts per minute per 50 ug protein - 5B, 6E, 7F) and those with a high total labelling ($\geq$ 1 x 10^6 counts per minute per peak 5C, 5D, 8C). In order to perform peptide "mapping" on these proteins and also to raise antisera against them, it was necessary to remove all traces of impurities. Peaks 6E and 7F were not further purified as they were present in small quantities and were estimated to be >95% pure. However, peaks 5B, 5C, 5D and 8C were applied separately to a column of hydroxyapatite (2.2 cm x 13 cm) equilibrated with buffer H and eluted with a 0-500 mM potassium phosphate linear gradient (150 ml) buffer I. The peaks were then pooled as shown in Figure 3.35. Further purification of peaks 5B and 5C was necessary and this was

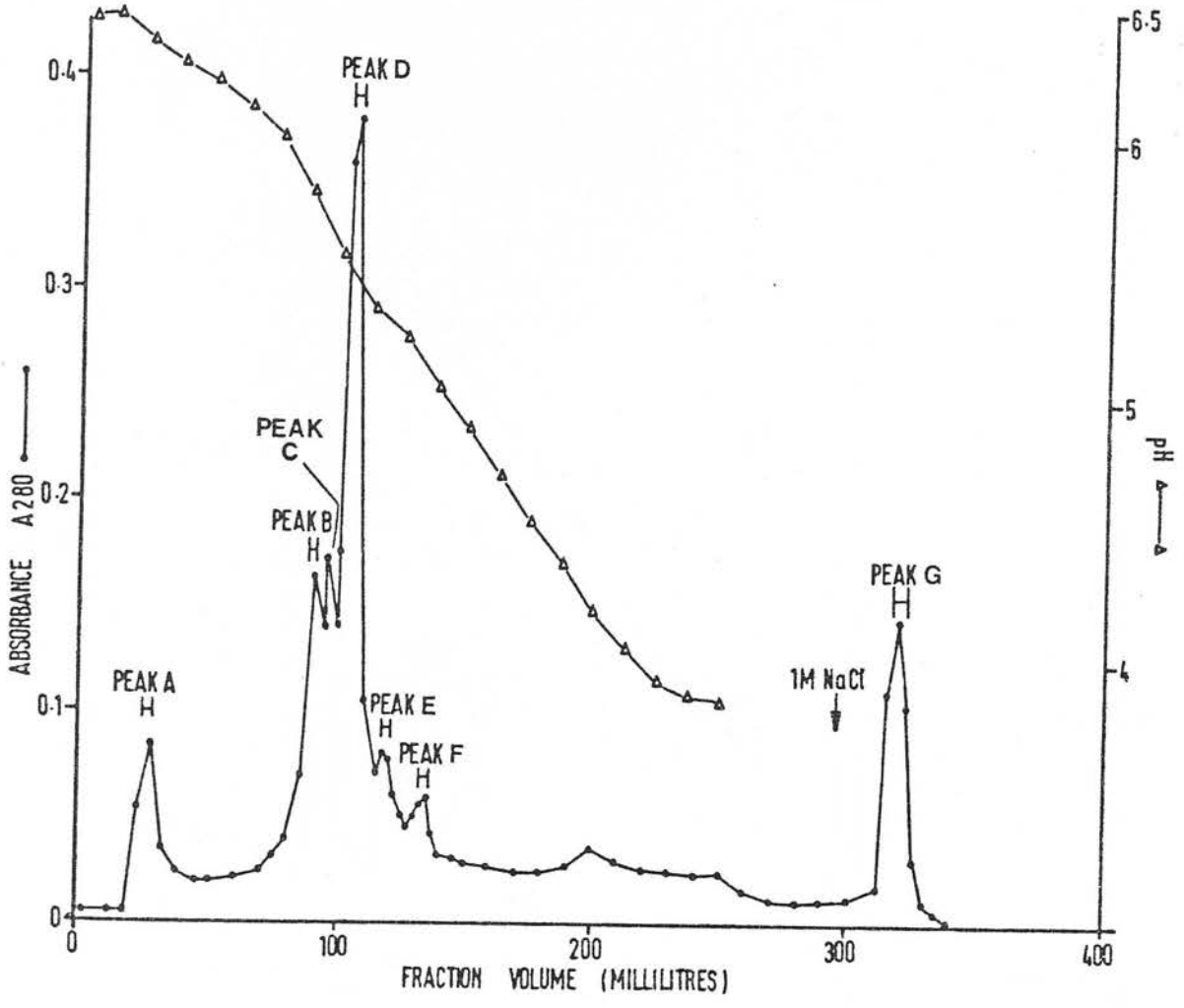


FIGURE 3.29 Chromatofocusing of Y' peak 5 from DEAE Sephadex. Peak 5 from DEAE (9 ml, 9 mg of protein) was applied to a column, 1 cm x 28 cm (22ml), of PBE 94 equilbrated with buffer C, and eluted with buffer D. Flow rate was 15.2 ml/h and the fraction volume 2.5 ml.

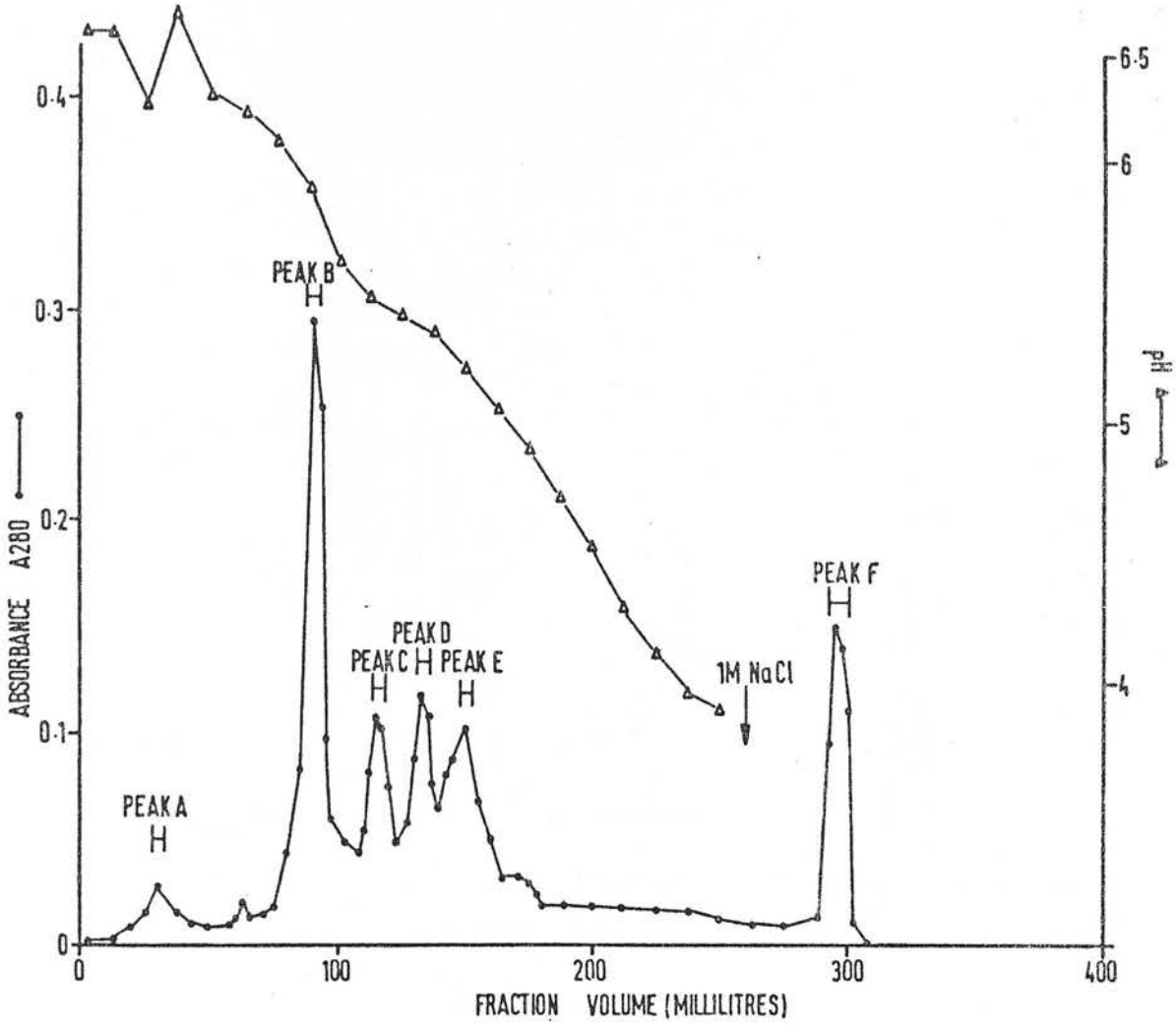


FIGURE 3.30 Chromatofocusing of Y' peak 6 from DEAE Sephadex. Peak 6 from DEAE (11 ml, 9 mg) was applied to a column, 1 cm x 28 cm (22 ml), of PBE 94, equilibrated with buffer C, and eluted with buffer D. Flow rate was 15.2 ml/h and the fraction volume was 2.5 ml.

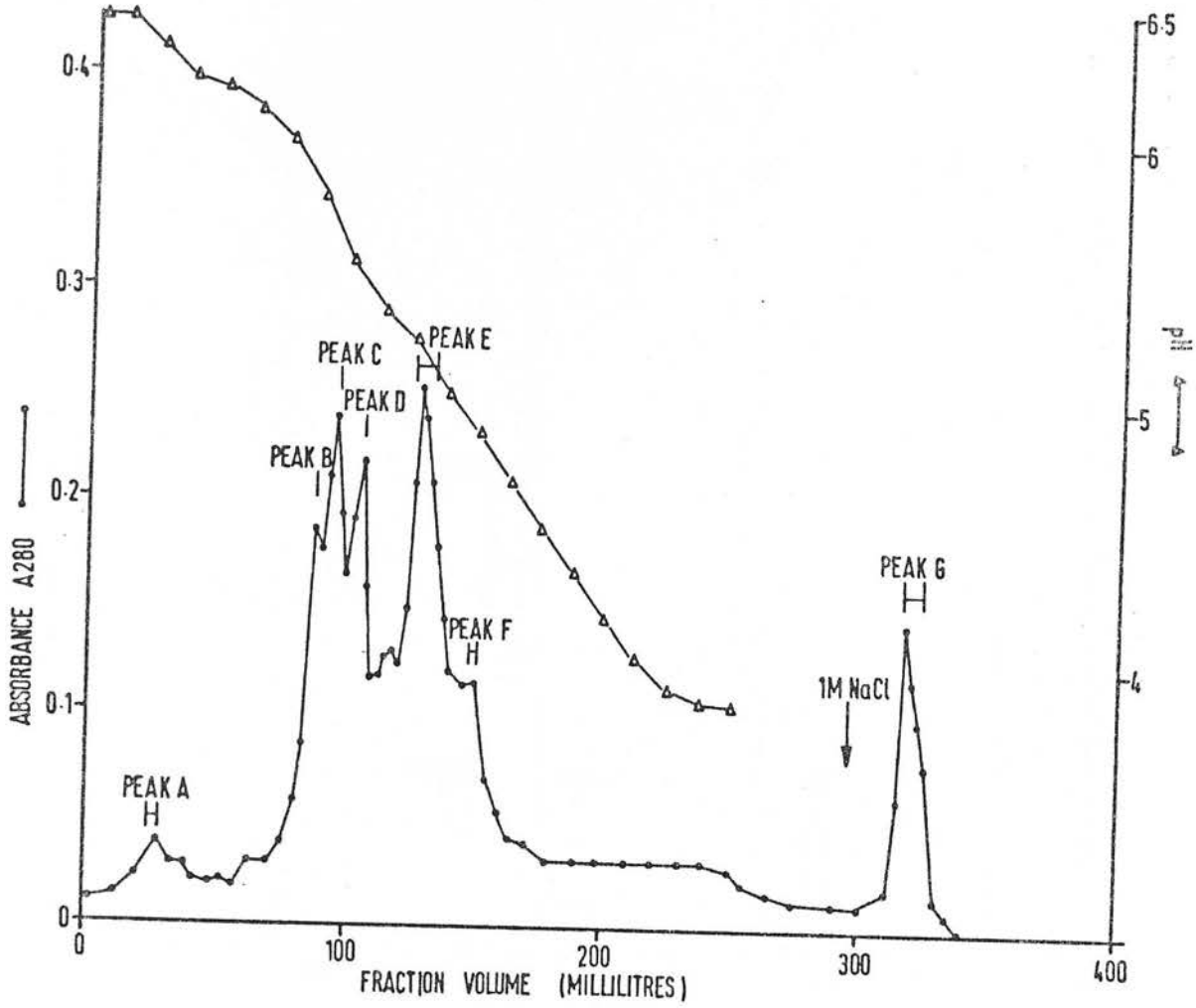


FIGURE 3.31 Chromatofocusing of Y' peak 7 from DEAE Sephadex. Peak 7 from DEAE (9 ml, 12 mg) was applied to a column, 1 cm x 28 cm (22ml), of PBE 94 equilibrated with buffer C, and eluted with buffer D. Flow rate was 15.2 ml/h and the fraction volume 2.5 ml.

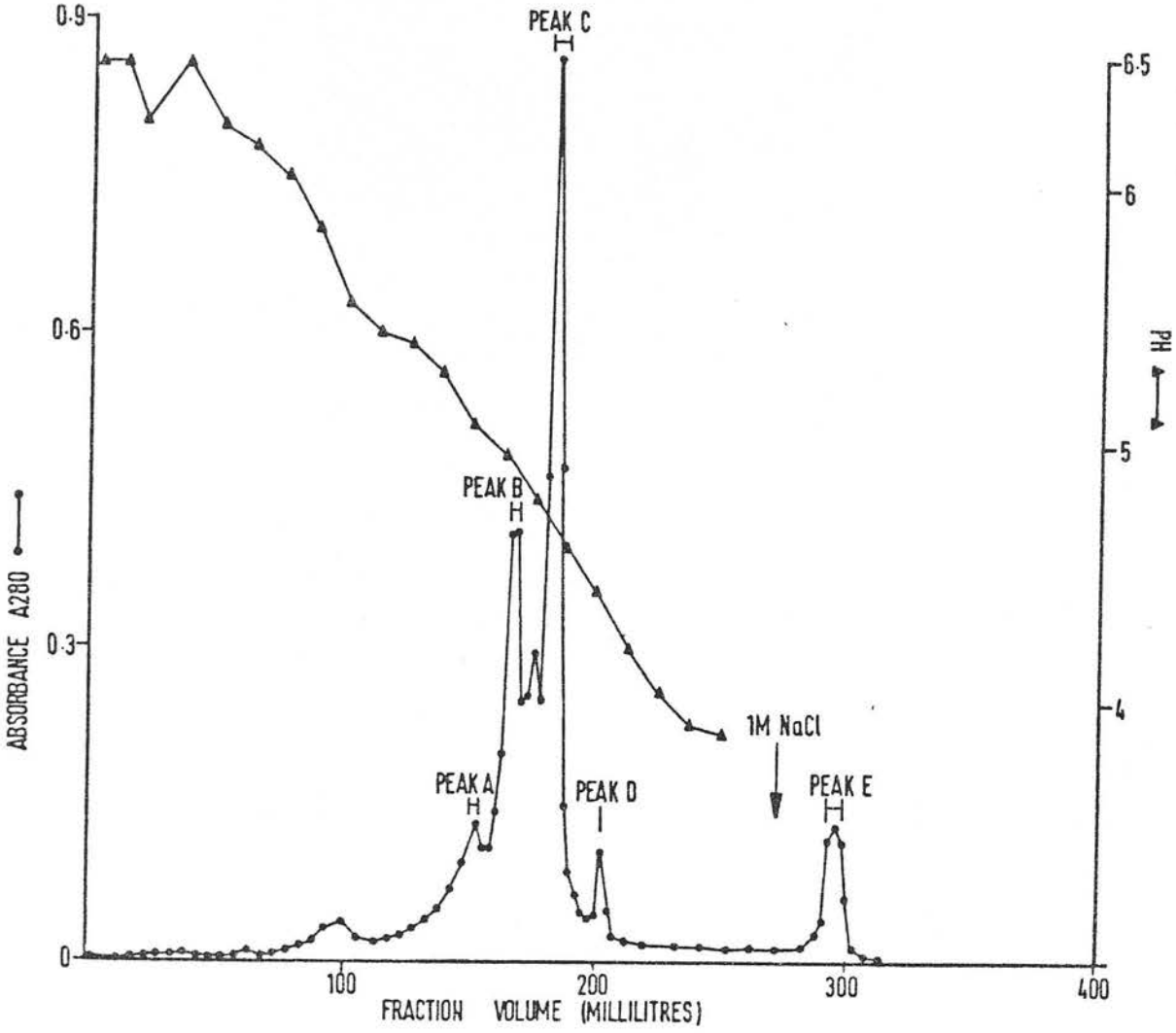


FIGURE 3.32 Chromatofocusing of Y' peak 8 from DEAE Sephadex. Peak 8 from DEAE (10 ml, 15.5 mg) was applied to a column, 1cm x 28 cm (22 ml), of PBE 94 equilibrated with buffer C, and eluted with buffer D. Flow rate was 15.2 ml/h and the fraction volume 2.5 ml.

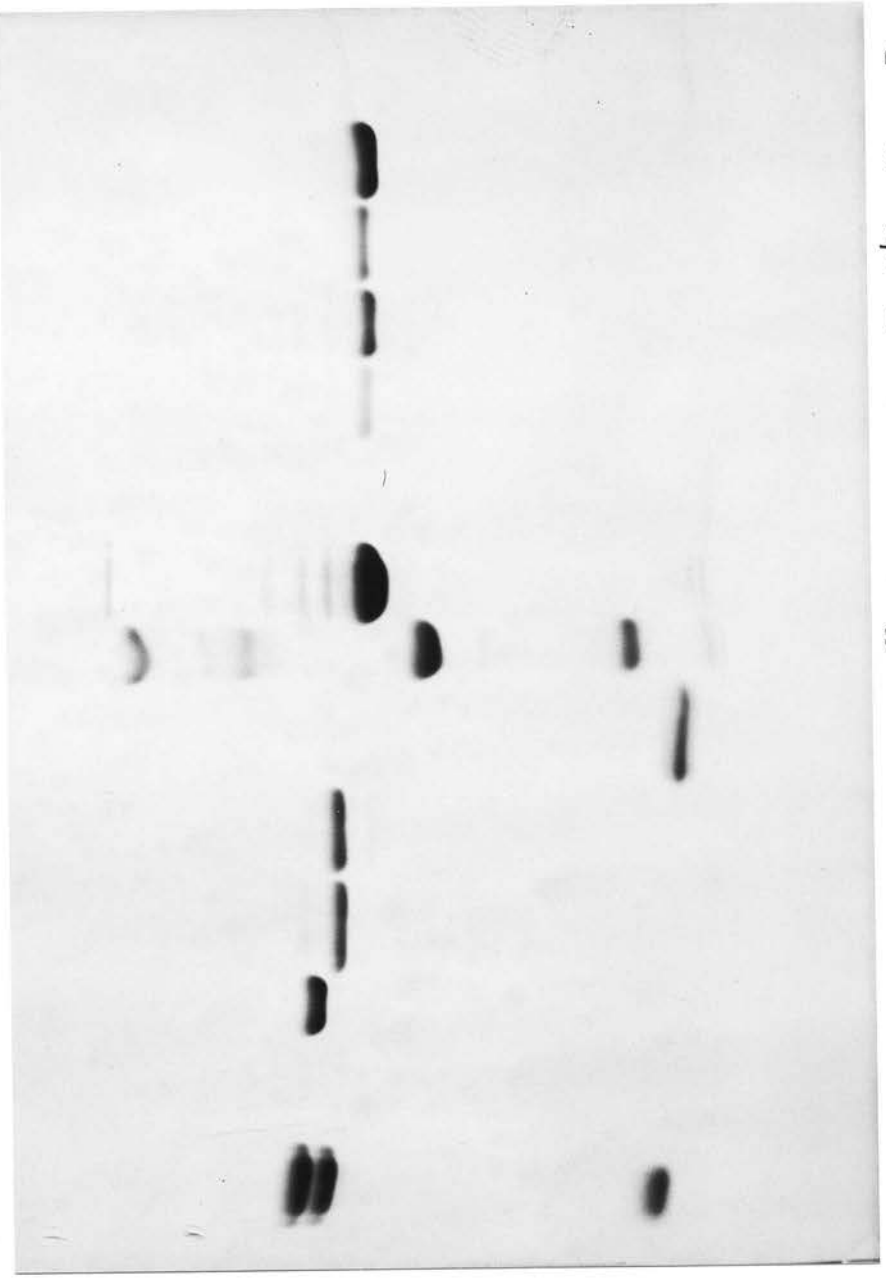

Sample: pk6 A B C D E marker pk8 A B B/C C D

FIGURE 3.33 SDS/polyacrylamide-gel electrophoresis of chromatofocusing fractions from peaks 6 and 8.

The fractions shown below were prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel. The sample lettering refers to Figures 3.30 and 3.32 (NOTE: B/C refers to the peak between B and C in peak 8).

Marker contained four proteins as standards - bovine serum albumin, ovalbumin, myoglobin and chymotrypsinogen A.

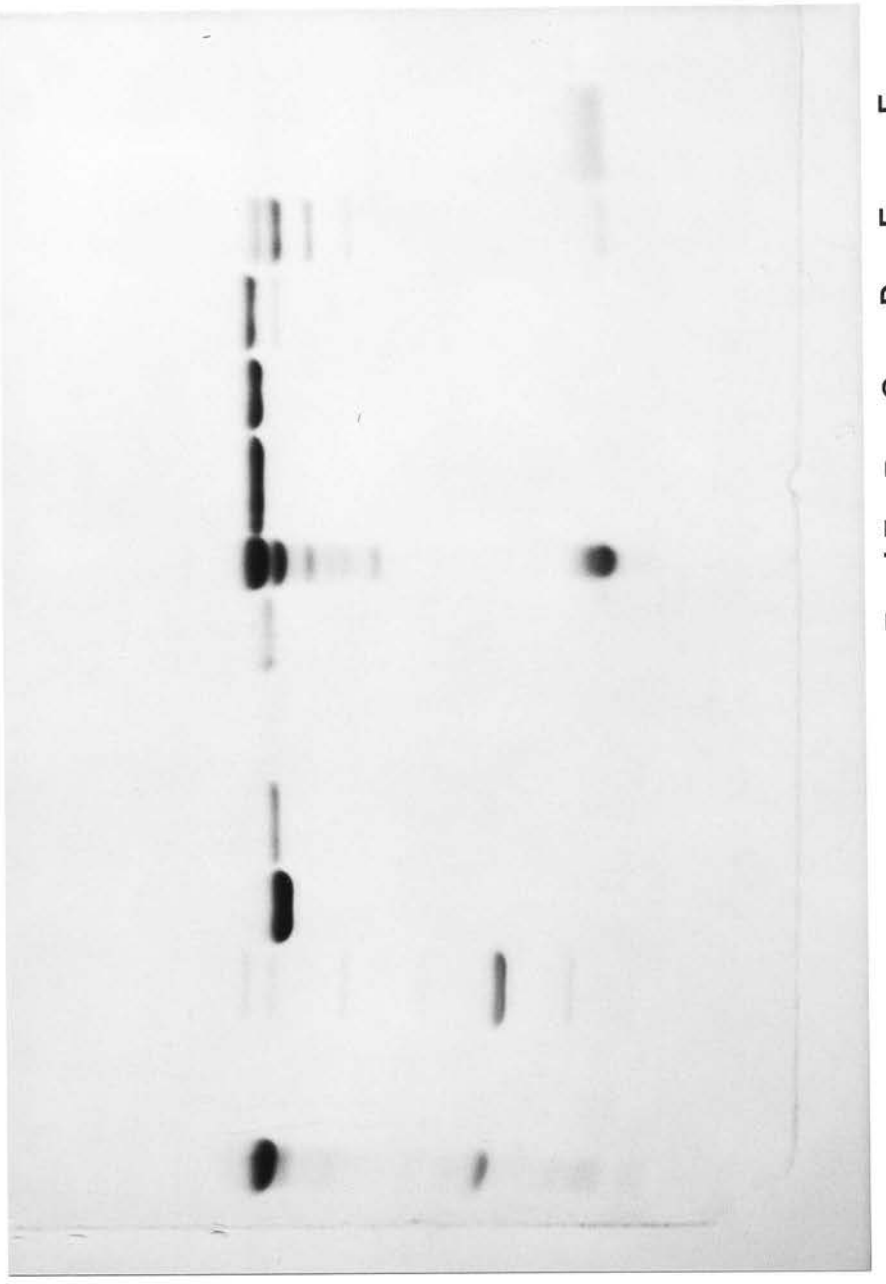

Sample:pk5 A B C D E F pk7 B C D E F

FIGURE 3.34 SDS/polyacrylamide-gel electrophoresis of chromatofocusing fractions from peaks 5 and 7.

The fractions shown below were prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel. The sample lettering refers to Figures 3.29 and 3.31.

Table 3.5: Molecular masses of proteins from chromatofocusing of Y' peaks 5, 6, 7 and 8

	Peak	Molecular mass (Da)
5	A	14 300
	B	19 600
	C	36 200
	D	36 200
	E	36 200
	F	*
	G	ND
6	A	*
	B	35 800
	C	35 800
	D	35 800
	E	15 600
	F	ND
7	A	ND
	B	39 000
	C	39 000
	D	39 000
	E	35 800
	F	14 900
	G	ND
8	A	33 000
	B	33 000
	C	33 000
	D	ND
	E	*

The approximate molecular masses given here are derived from the gels in Figures 3.30 and 3.31. A 12% resolving gel was used, with a four protein marker as standard.

ND = Not detected; * - insufficient material.

achieved by re-chromatofocusing with a shallower pH gradient (Figure 3.36) and the peaks pooled as shown. Analysis of the purified peaks by SDS/polyacrylamide-gel electrophoresis (Figure 3.37) illustrated the purity of the proteins.

The purification procedure for Y' proteins is shown in Table 3.6.

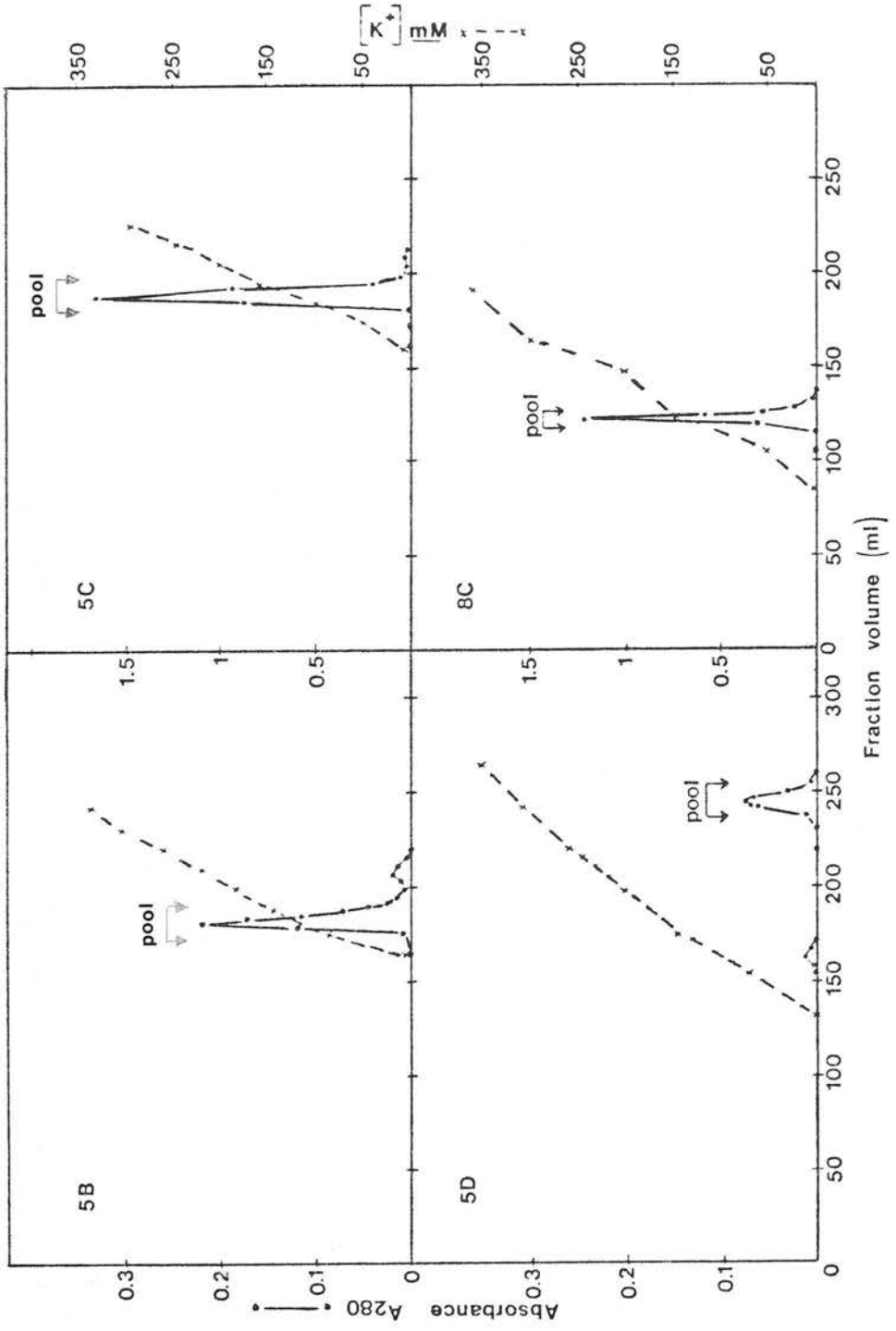

5B
5C
5D
8C
pool
Absorbance A280
[K+] mM
Fraction volume (ml)

FIGURE 3.35 Purification of peaks 5B, 5C, 5D and 8C on hydroxyapatite. Peaks 5B (2.75 mg), 5C (10.5 mg), 5D (5.6 mg) and 8C (6.9 mg) were applied to a column of hydroxyapatite (22 cm x 13 cm) equilibrated with buffer H and eluted with a 0-500 mM potassium phosphate linear gradient (150 ml buffer I). Flow rate was 13 ml/h and the fraction volume 2.1 ml. Peaks were pooled as shown.

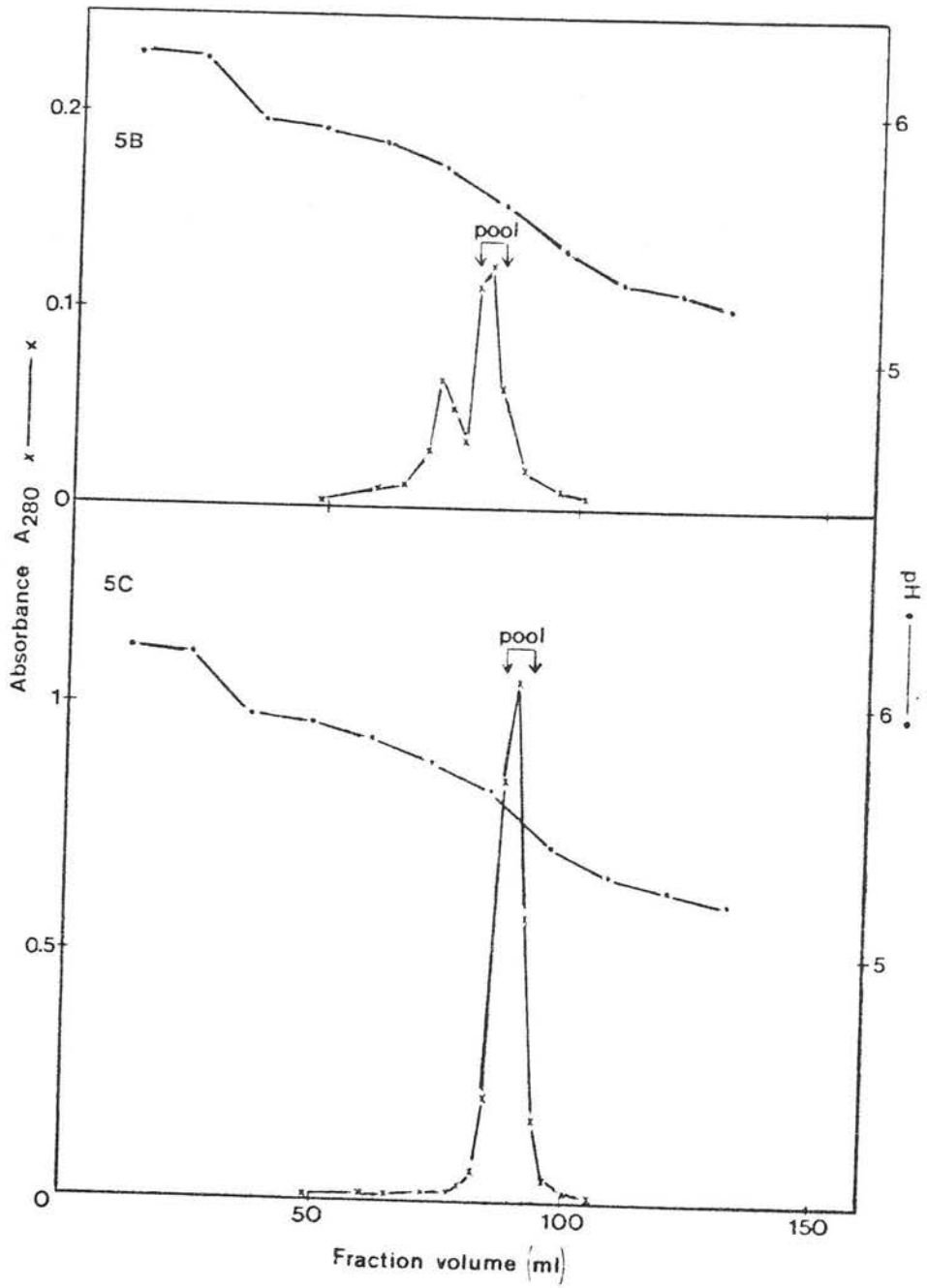

5B
pool
5C
pool
0.2
0.1
0
1
0.5
0
6
5
6
5
50
100
150
Absorbance A_{280} x — x
pH • — •
Fraction volume (ml)

FIGURE 3.36 Purification of peaks 5B and 5C by re-chromatofocusing. Peaks 5B (1.4 mg) and 5D (8.2 mg) were applied to a column of PBE 74 (1 cm x 28 cm) and equilibrated with buffer C (pH 6.2). The protein was then eluted with buffer D (pH 4.8). Flow rate was 14.5 ml/h and fraction volume 2.4 ml. Peaks were pooled as shown.

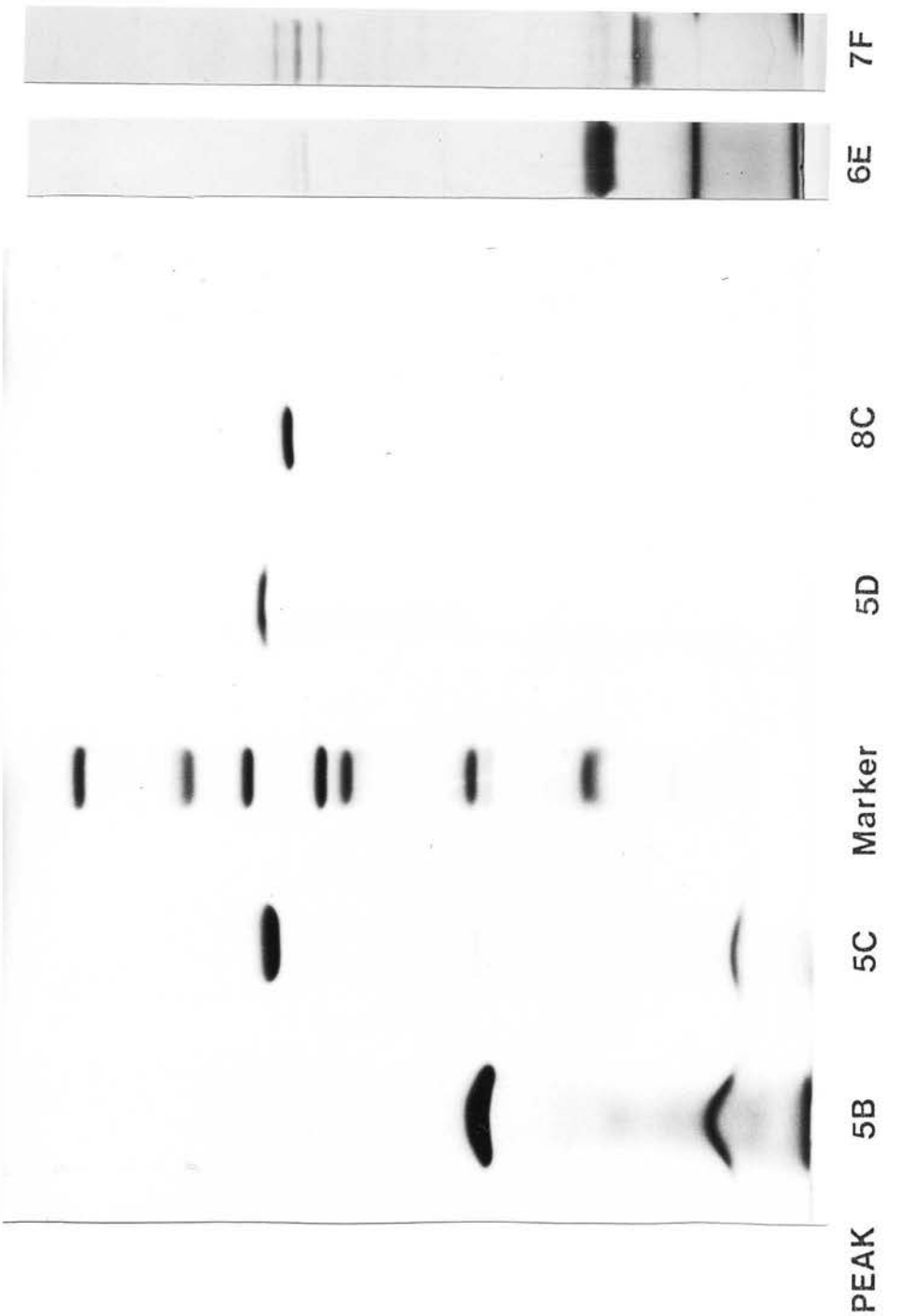

7F
6E
8C
5D
Marker
5C
5B
PEAK

FIGURE 3.37 SDS/polyacrylamide-gel electrophoresis of pure Y' proteins. Samples were prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel. The marker contained seven proteins as standards and a typical calibration is shown in Figure 2.1.

The streaks at the bottom of some tracks are due to polybuffer present in the sample.

Photoaffinity labelling of Y' proteins

At each stage during the purification of Y' proteins a portion (50 µg) was subjected to photoaffinity labelling. Table 3.6, which summarises the purification procedure, includes the results of these experiments.

As a first experiment, portions of each peak from DEAE Sephadex were photoaffinity labelled with [^{125}I]-3β azidocholylhistamine for up to 60 min, and after TCA precipitation and extensive washing, the products were analysed by SDS/polyacrylamide-gel electrophoresis. The results are shown in Figure 3.38. It can clearly be seen that only peaks 5, 6, 7 and 8 have any substantial amounts of labelling; it was this result which prompted the further investigation of these peaks.

Subsequent photoaffinity labelling experiments, however, utilised less protein, a shorter photolysis time and a different, faster, separation method for analysis of the covalent incorporation of [^{125}I]. A small pilot experiment with peak 5B, thought to be a bile acid-binding protein, confirmed the validity of this approach and is shown in Figure 3.39. With lower concentrations of protein e.g. 5 µg/ml instead of 1-10 mg/ml, a shorter photolysis time can be employed. The optimal time, for maximal covalent incorporation of [^{125}I], as indicated in Figure 3.39 was 15 min. TCA precipitation alone was found to be insufficient for the removal of all non-covalently bound label, even with extensive washing

TABLE 3.6 PURIFICATION PROCEDURE FOR Y' PROTEINS

Fraction	Volume (ml)	Protein (A_{280})	[Na+] DEAE (mM)	GST activity (A_{340})	Isoelectric point	Molecular mass (Da)	Covalent Incorp. [^{125}I] (CPMx10-3/50µg)	Total covalent [^{125}I] Incorp. (CPMx10-6)	Yield (%)
RAT LIVER CYTOSOL ON -									
A) G-100	202	6121		460			27	3311	100
B) G-75 (1)	89	1502		286			19	596	18
C) G-75 (2)	39	544		116			36	392	12
D) DEAE	105	165		0.004			29	94	3
CHROMATOFOCUSING OF DEAE PEAK:									
5(Total)	9	9	75	0			45	8.1	0.25
A	5	0.325			6.3	14 300	15	0.1	0.003
B	5	0.79			5.75	19 600	113	1.8	0.054
C	5	2.5			5.65	36 200	20	1	0.03
D	7.5	2.25			5.45	36 200	23	1	0.03
E	5	0.39			5.25	36 200	20	0.2	0.005
F	5	0.25			5.05	*	*	*	*
G	7.5	1.16			SALT	ND	17	0.4	0.01
6(Total)	11	9	90	0			20	3.6	0.11
A	7.5	0.225			VOID	*	*	*	*
B	7.5	1.74			5.8	35 800	18	0.6	0.02
C	5	0.52			5.45	35 800	13	0.1	0.004
D	5	0.43			5.35	35 800	25	0.2	0.006
E	7.5	0.73			5.2	15 600	116	1.7	0.05
F	10	1.14			SALT	ND	8	10.2	0.005

TABLE 3.6 PURIFICATION PROCEDURE FOR Y' PROTEINS (contd.)

Fraction	Volume (ml)	Protein (A_{280})	[Na^+] DEAE (mM)	GST activity (A_{340})	Isoelectric point	Molecular mass (Da)	Covalent Incorp. [^{125}I] (CPMx10^{-3}/50µg)	Total covalent [^{125}I] Incorp. (CPMx10^{-6})	Yield (%)
CHROMATOFOCUSING OF DEAE PEAK:									
7(Total)	9	12	110	0			4	0.9	0.03
A	5	0.325			6.3	ND	15	0.1	0.003
B	2.5	0.44			5.75	39 000	17	0.2	0.004
C	2.5	0.56			5.6	39 000	19	0 2	0.007
D	2.5	0.51			5.4	39 000	17	0.2	0.005
E	10	2.1			5.15	35 800	8	0.3	0.001
F	5	0.5			4.85	14 900	99	1	0.03
G	7.5	0.87			SALT	ND	10	0.2	0.005
8(Total)	10	15.5	150	0			7	2.2	0.07
A	5	0.65			5.1	33 000	33	0.4	0.01
B	5	2.05			4.9	33 000	14	0.6	0.02
C	7.5	4.41			4.65	33 800	31	2.8	0.08
D	2.5	0.21			4.35	ND	10	0.04	0.001
E	7.5	0.13			SALT	*	*	*	*

NOTES: GST = Glutathione S-transferase; Isoelectric point from chromatofocusing; ND = not detected; * = insufficient material for analysis; Yield is expressed at each stage as percentage of total covalent incorporation.

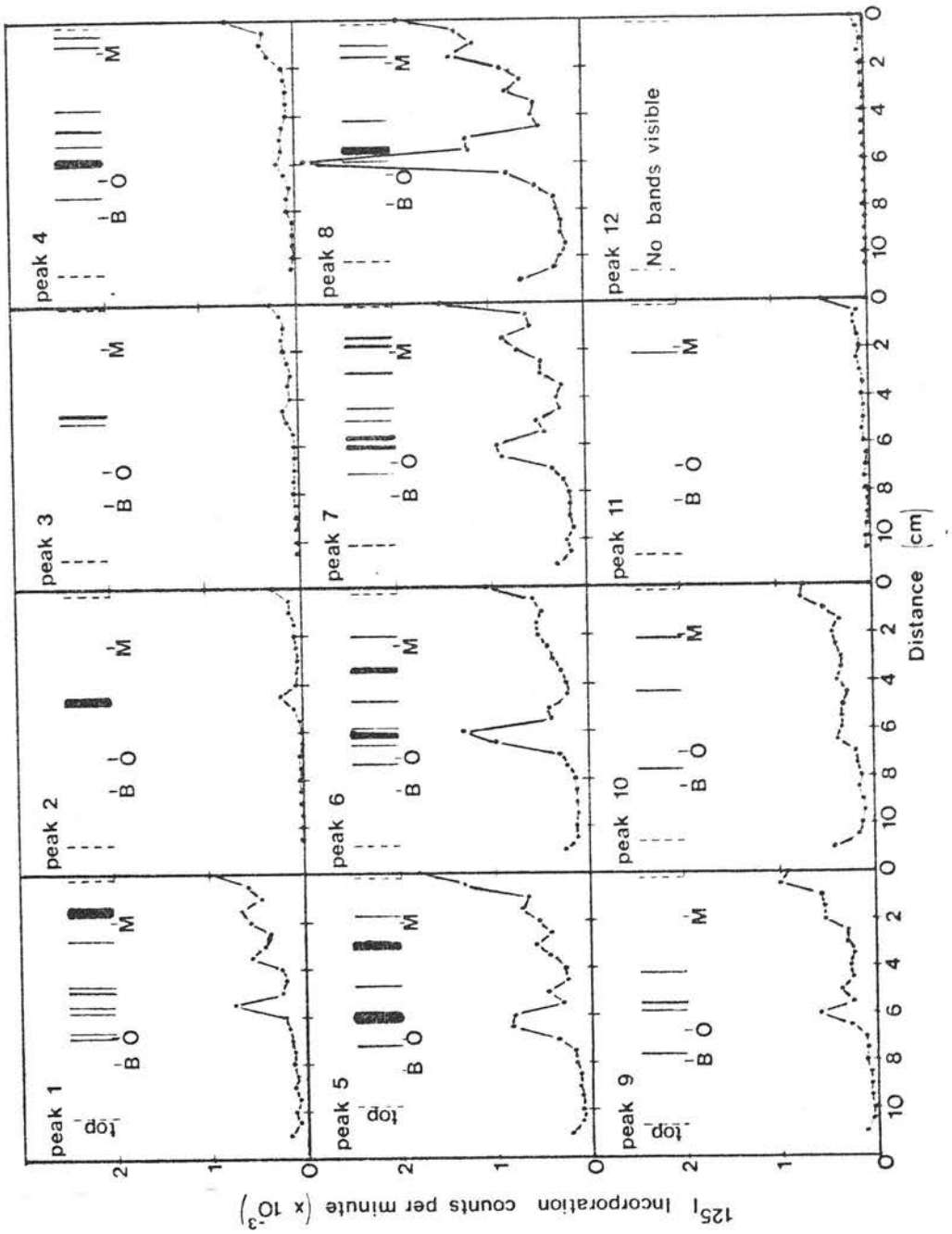

peak 1
peak 2
peak 3
peak 4
peak 5
peak 6
peak 7
peak 8
peak 9
peak 10
peak 11
peak 12
No bands visible
top
B
O
M
Distance (cm)
^{125}I incorporation counts per minute (x 10^{-3})

FIGURE 3.38 Photoaffinity labelling of peaks from DEAE Sephadex. Portions of each peak from DEAE Sephadex were photolysed with [^{125}I]-3β-azidocholylhistamine (10 μCi, 5 pmol) for 60 min. The protein was precipitated with 10% TCA, prepared for SDS/polyacrylamide-gel electrophoresis and analysed on a 12% resolving gel. After staining, the gel tracks were cut into 0.5 cm slices and counted for [^{125}I].

Broken lines indicate top and bottom of resolving gel; an attempt has been made to represent the various protein bands in accordance with their original appearance.

B-bovine serum albumin; O-ovalbumin; M-myoglobin.

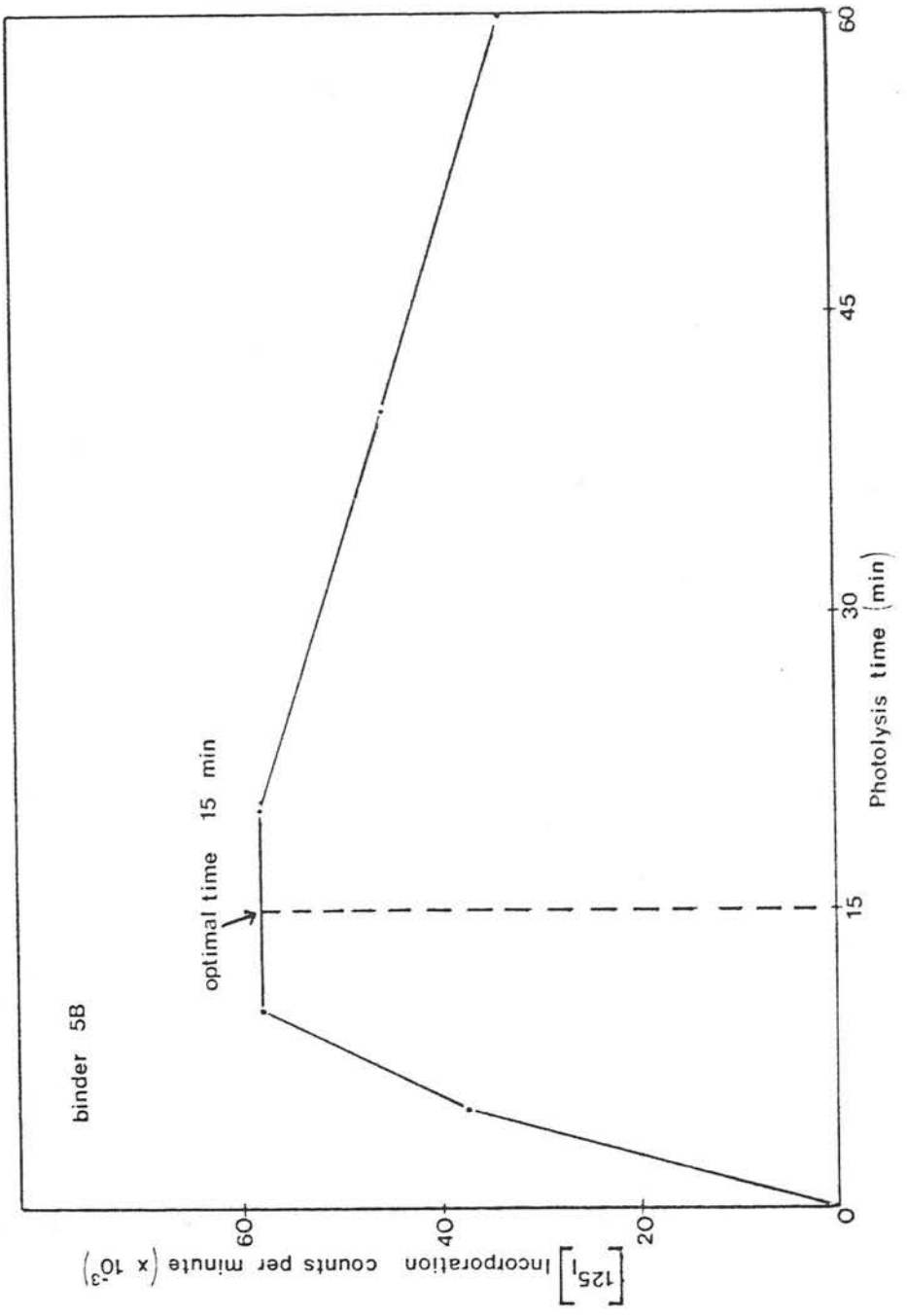

binder 5B
optimal time 15 min
[125I] Incorporation counts per minute (x 10-3)
0
20
40
60
15
30
45
60
Photolysis time (min)

FIGURE 3.39 Photoaffinity labelling of peak 5B at low protein concentration (50 µg) with [^{125}I]-3β azidocholylhistamine (2.5 µCi, 1.25 pmol) in buffer A (10 ml) for the times shown below. The samples were precipitated with TCA, extensively washed, then taken up in buffer A containing 1% SDS (w/v) (500 µl). Samples were run on a column (1.5 cm x 20 cm) of Sephadex G-25 fine and eluted with the same buffer. Flow rate was 25 ml/h and fraction volume 4 ml.

(Table 3.7). A quicker method, capable of handling more samples, was employed. This involved gel filtration of the labelled proteins under denaturing conditions, i.e. in buffer A containing 1% SDS (w/v). With the protein structure in random coil formation, all non-covalently bound label was separated from labelled protein, the former eluting from the column in the salt volume, the latter in the void volume. The figures given in Table 3.6 are derived from analysis of the labelled proteins using this method. The Y' peaks from DEAE Sephadex were also analysed in this way following photoaffinity labelling, and the results are shown in Table 3.8 for comparison with the electrophoretic analysis (Figure 3.38).

PEPTIDE "MAPPING" OF BILE ACID-BINDING PROTEINS

Peaks 5B, 5C, 5D, 6E, 7F and 8C from chromatofocusing were analysed by peptide "mapping". These proteins had been identified as putative bile acid-binding proteins from the results of photoaffinity labelling experiments, and could be split into two categories:-

1) Proteins with a high specific labelling ($\geq 1 \times 10^5$ counts per minute per 50 ug protein) - 5B, 6E and 7F;
2) Proteins with a high total labelling ($\geq 1 \times 10^6$ counts per minute) - 5C, 5D, 8C.

Peptide "mapping" was carried out using two different methods - by limited proteolytic digestion in the presence of SDS and analysis on SDS/PAGE (Cleveland gels), and by

Table 3.7 Comparison of TCA precipitation and TCA precipitation/denaturing gel filtration for analysis of labelled proteins

Fraction	[^{125}I] CPM x 10-3/50 µg protein	
	TCA Precipitation	TCA precipitation/denaturing gel filtration
Peak 5B	213	34
Peak 5D	118	8

Peaks 5B and 5D (50 µg each) were photoaffinity labelled with [^{125}I]-3β-azidocholylhistamine (2.5 µCi, 1.25 pmol). Following TCA precipitation, the protein samples were counted for [^{125}I], before application to a column of G-25 Sephadex fine, 1.5 cm x 20 cm, and eluted with buffer A containing 1% SDS (w/v). Fraction volume was 4 ml and the flow rate 25 ml/h. The labelled protein eluted from the column in the void volume (approximately 10 ml) and the non-covalently-bound label in the salt volume (approximately 25 ml).

Table 3.8 Photoaffinity labelling of Y' peaks from DEAE Sephadex and analysis by TCA precipitation/denaturing gel filtration.

Peak	[^{125}I] counts per minute (x 10-3)/50 µg
1	24
2	17
3	11
4	17
5	53
6	50
7	49
8	30
9	11
10	19
11	*
12	*

Portions (50 µg) of each Y' peak from DEAE Sephadex were photoaffinity labelled with [^{125}I]-3βazidocholylhistamine (2.5 µCi, 1.25 pmol) in buffer A (10 ml) for 15 min. The samples were precipitated with 10% TCA, extensively washed, then taken up in buffer A (500 µl) containing 1% SDS (w/v). Samples were then eluted from a column (1.5 cm x 20 cm) of Sephadex G-25 fine with the same buffer. Flow rate was 25 ml/h and fraction volume 4 ml. * = analysis not performed.

tryptic digestion and analysis on reverse-phase high performance liquid chromatography.

Limited proteolytic digestion in the presence of SDS

The peptide "maps" derived from limited digestion by -chymotrypsin are shown in Figures 3.40 and 3.41. The "maps" of 5B, 6E and 7F show that these proteins are somewhat resistant to digestion, with only a small number of peptides being generated even at a protease concentration of 100 µg/ml. Two, or possibly three, peptides, of approximate molecular masses 10 000 Da, 11 000 Da and 15 000 Da, may be common to these three proteins, and the latter perhaps also to 5C, 5D and 8C. However, as these bands run close to the lower molecular mass component of α-chymotrypsin, a difficulty arises in distinguishing between them. These are indicated in Figure 3.40.

Proteins 5C, 5D and 8C digested somewhat better (Figure 3.41) and show two peptides apparently common to all three, with approximate molecular masses of 18 000 Da and 15 000 Da. These bands are distinct from those of α-chymotrypsin.

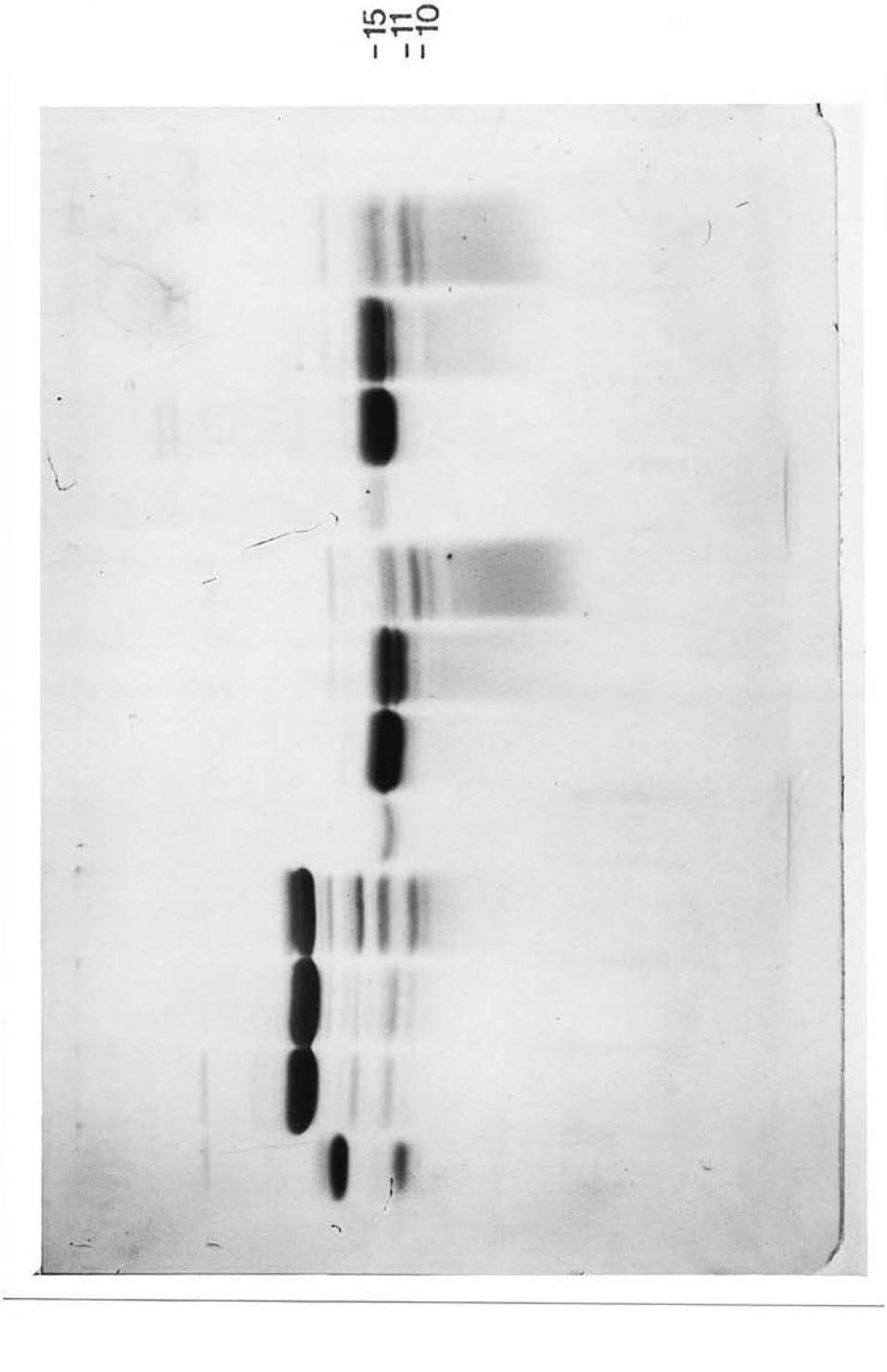

-15
-11
-10
TRACK: 1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 3.40 Peptide "maps" of peaks 5B, 6E and 7F by limited proteolytic digestion in presence of SDS.

A portion (100 µg) of each protein was partially digested in 0.5% SDS (w/v) for 60 min at 37°C in the presence of either 100 µg/ml, 12.5 µg/ml or 2.5 µg/ml of α-chymotrypsin. The reaction was stopped by boiling after the addition of SDS and mercaptoethanol to final concentrations of 2% and 10% respectively. The samples were then analysed by SDS/polyacrylamide-gel electrophoresis on 16.5% resolving gel.

The samples applied were 1: α-chymotrypsin (10 µg), 2-4: binder 5B and 2.5, 12.5, 100 µg/ml α-chymotrypsin respectively, 5-8: binder 6E (2µg) and 2.5, 12.5, 100 µg/ml α-chymotrypsin respectively, 9-12: binder 7F (2µg) and 2.5, 12.5, 100 µg/ml α-chymotrypsin respectively.

α-chymotrypsin, treated in the same way as the Y' binders, was dissociated into its subunits (approximate molecular masses 17 000 Da and 11 000 Da respectively) and used as a molecular mass marker (Track 1).

Common peptides are indicated, with approximate molecular masses in Da ($\times 10^{-3}$).

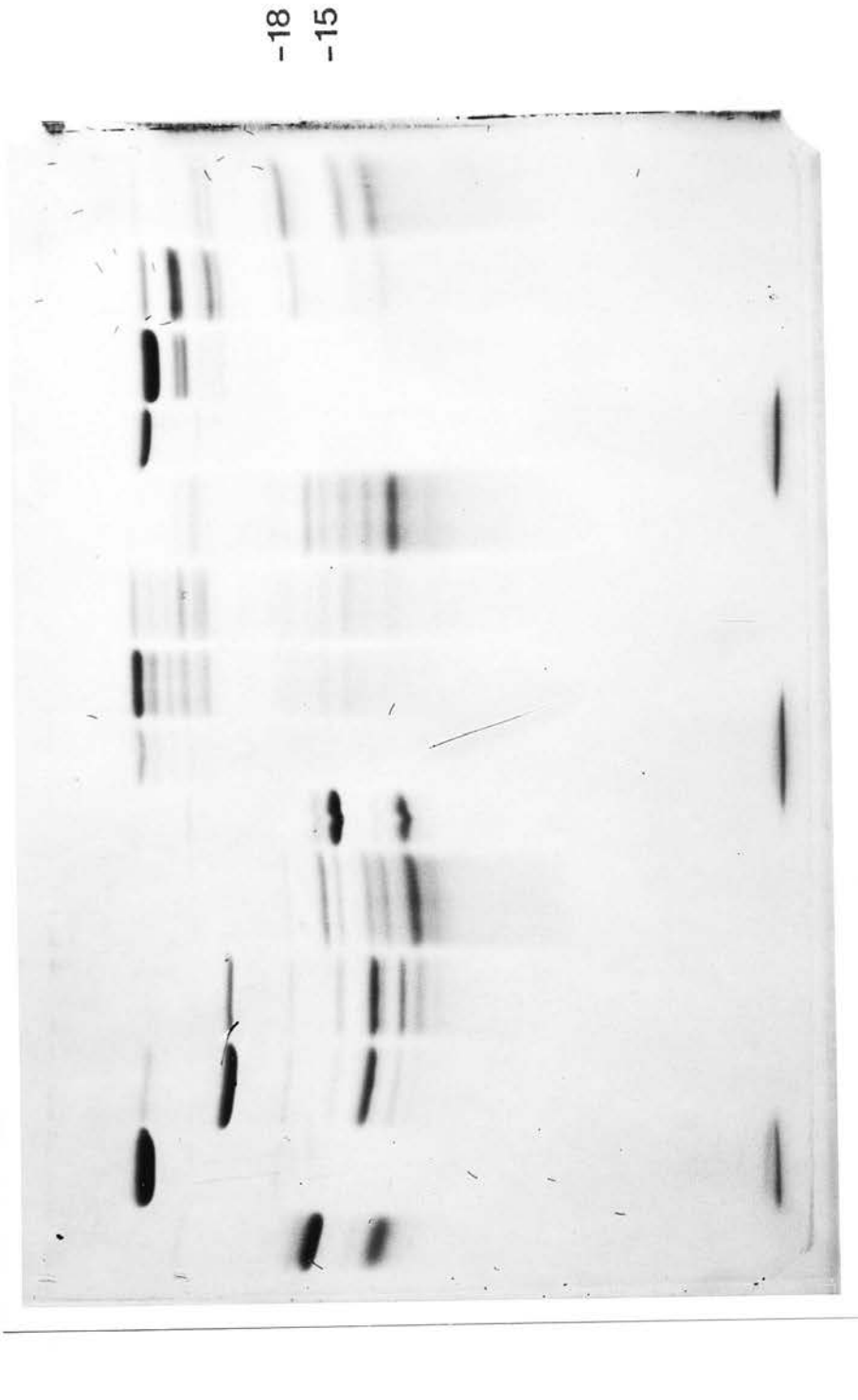

-18
-15
TRACK: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 3.41 Peptide "maps" of peaks 5D and 8C by limited proteolytic digestion in the presence of SDS.

A portion (100 µg) of each protein was partially digested in 0.5% SDS (w/v) for 60 min at 37°C in the presence of either 100 µg/ml, 12.5 µg/ml or 2.5 µg/ml of α-chymotrypsin. The reaction was stopped by boiling after the addition of SDS and mercaptoethanol to final concentrations of 2% and 10% respectively. The samples were then analysed by SDS/polyacrylamide-gel electrophoresis on 16.5% resolving gel.

The samples applied were 1: α-chymotrypsin (10µg), 2-5: binder 5C (10µg) and 2.5, 12.5, 100 µg/ml α-chymotrypsin respectively, 11-14: binder 8C (5µg) and 2.5, 12.5, 100 µg/ml α-chymotrypsin respectively.

α-chymotrypsin, treated in the same way as the Y' binders, was dissociated into its subunits (approximate molecular masses 17 000 Da and 11 000 Da respectively) and was used as a molecular mass marker.

Common peptides are indicated, with approximate molecular masses in Da (x 10^{-3}).

Tryptic digestion and analysis on reverse-phase high performance liquid chromatography

The peptide "maps" of the six Y' proteins and four glutathione S-transferases are shown to Figures 3.42 to 3.51 and Tables 3.9 to 3.11 summarises the results.

The dried, digested proteins were taken up in a volume of starting solvent (water and trifluoroacetic acid (TFA), 0.05% (v/v)) such that the resulting solution was approximately 1 mg/ml with respect to protein concentration. The solution was centrifuged (1500 g, 15 min) immediately prior to application to the HPLC system in order to remove insoluble material which was present in all the digests.

In addition, each of the ten bile acid-binding proteins were photoaffinity labelled and then peptide "mapped" as previously described. Fractions were collected from the column and counted for radioactivity. Figures 3.52 to 3.54 show these results, with a summary in Tables 3.12 to 3.14. A portion of unreacted [^{125}I]-3β azidocholylhistamine was eluted from the reverse-phase column under the same conditions as for the peptide "maps" in order to determine the retention time (Rt) of the free molecule.

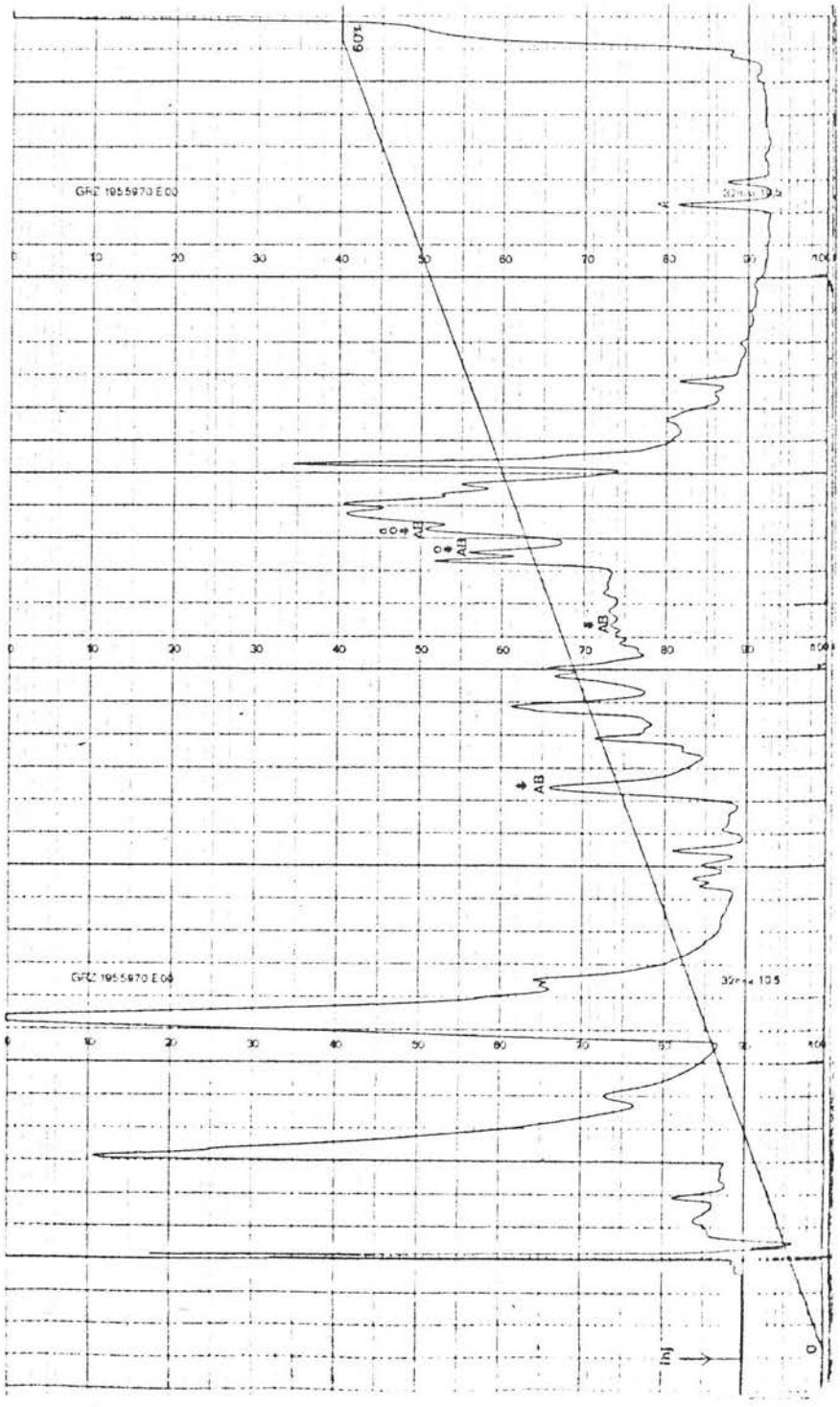

60°
GRF 1955970 E00
10
20
30
40
50
60
70
80
AB
AB
AB
AB
GFZ 1955970 E06
32m-4 10 5
[m]
0

FIGURE 3.42 Peptide "map" of binder 5B.

A portion of binder 5B (500 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (25 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile.

The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - A: 5B - 6E

B: 5B - 7F

C: 6E - 7F

*: common to group 1 binders

o: common to group 1 and group 2 binders

△: common to Y' binders and glutathione S-transferases AA, A, D and F.

INJ: injection.

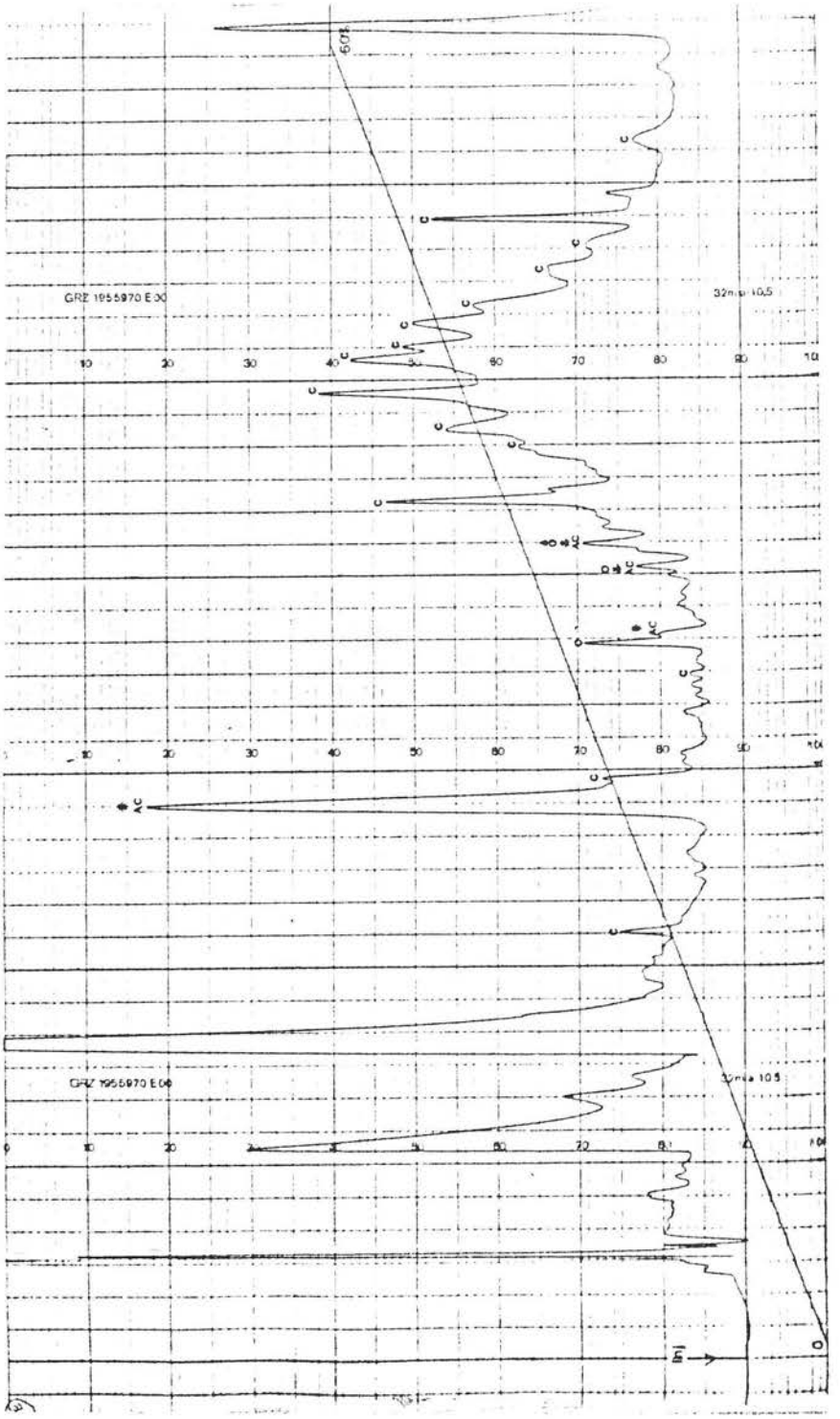

GRZ 1955970 E 00
GRZ 1955970 E 04

FIGURE 3.43 Peptide "map" of binder 6E.

A portion of binder 6E (200 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (70 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm. Key to common peaks - See legend to Figure 3.42.

INJ: injection.

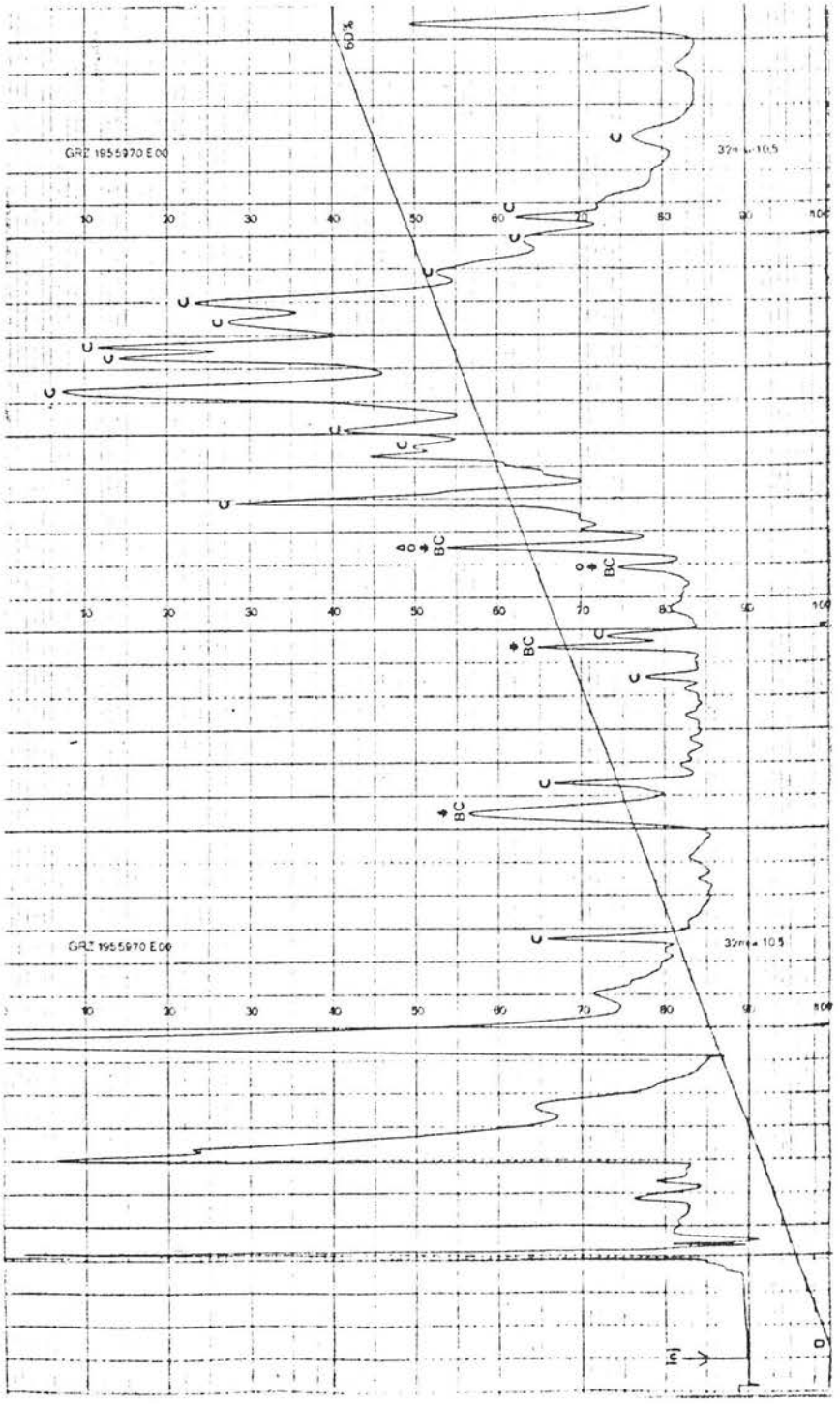

60%
GRZ 1955970 E 00
32m x 10.5
C
BC
10
20
30
40
50
60
70
80
90
100
GRZ 1955970 E 09
32m x 10.5
Inj
D

FIGURE 3.44 Peptide 'map" of binder 7F.

A portion of binder 7F (600 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (50 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm. Key to common peaks - See legend to Figure 3.42.

INJ: injection.

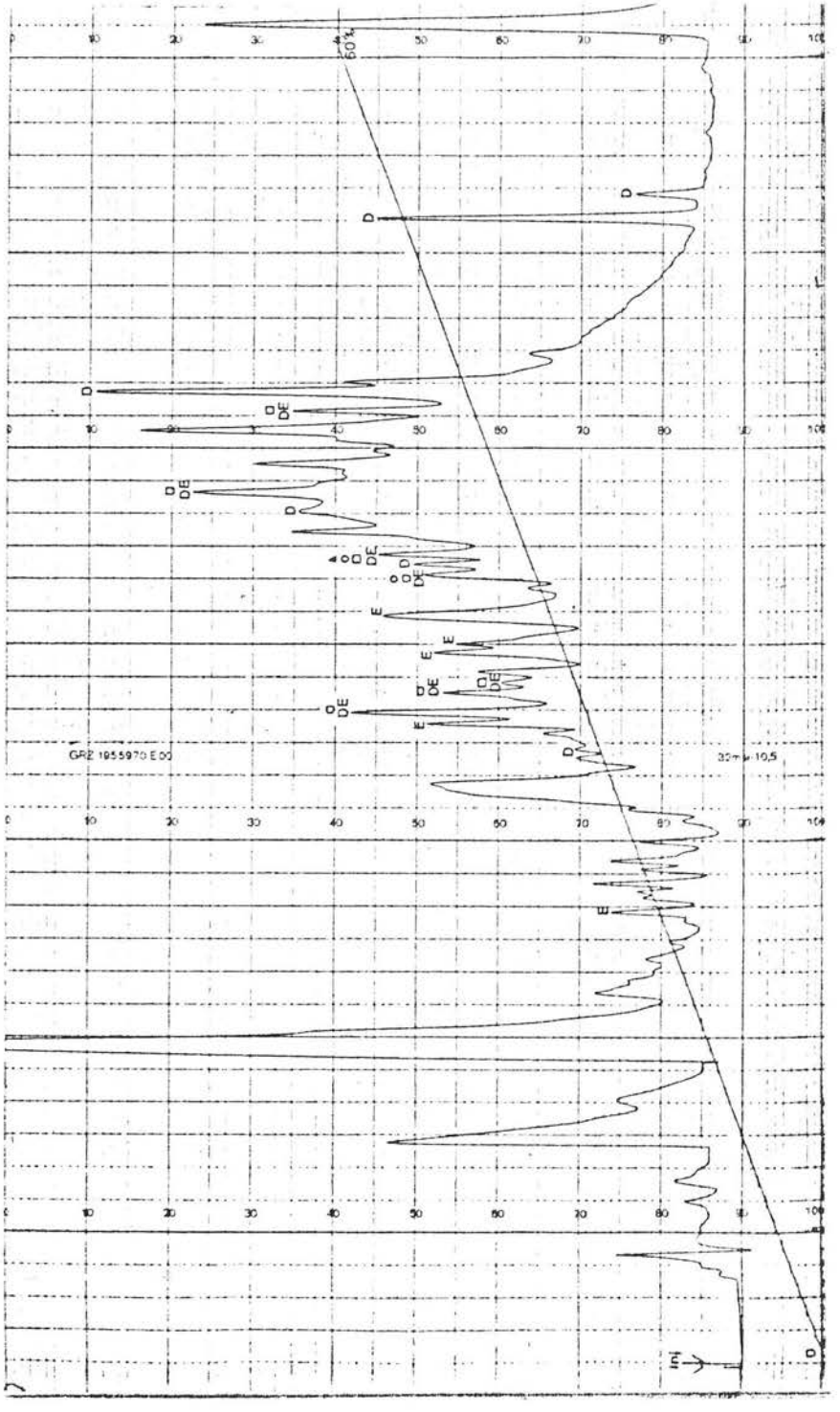

60%
GRZ 1955970 E 00
30 → 10,5
inj

FIGURE 3.45 Peptide "map" of binder 5C.

A portion of binder 5C (500 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (100 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - D: 5C - 5D

E: 5C - 8C

F: 5D - 8C

□ : common to group 2 binders

o : common to group 1 and group 2 binders

△ : common to Y' binders and glutathione S-transferases.

INJ: injection.

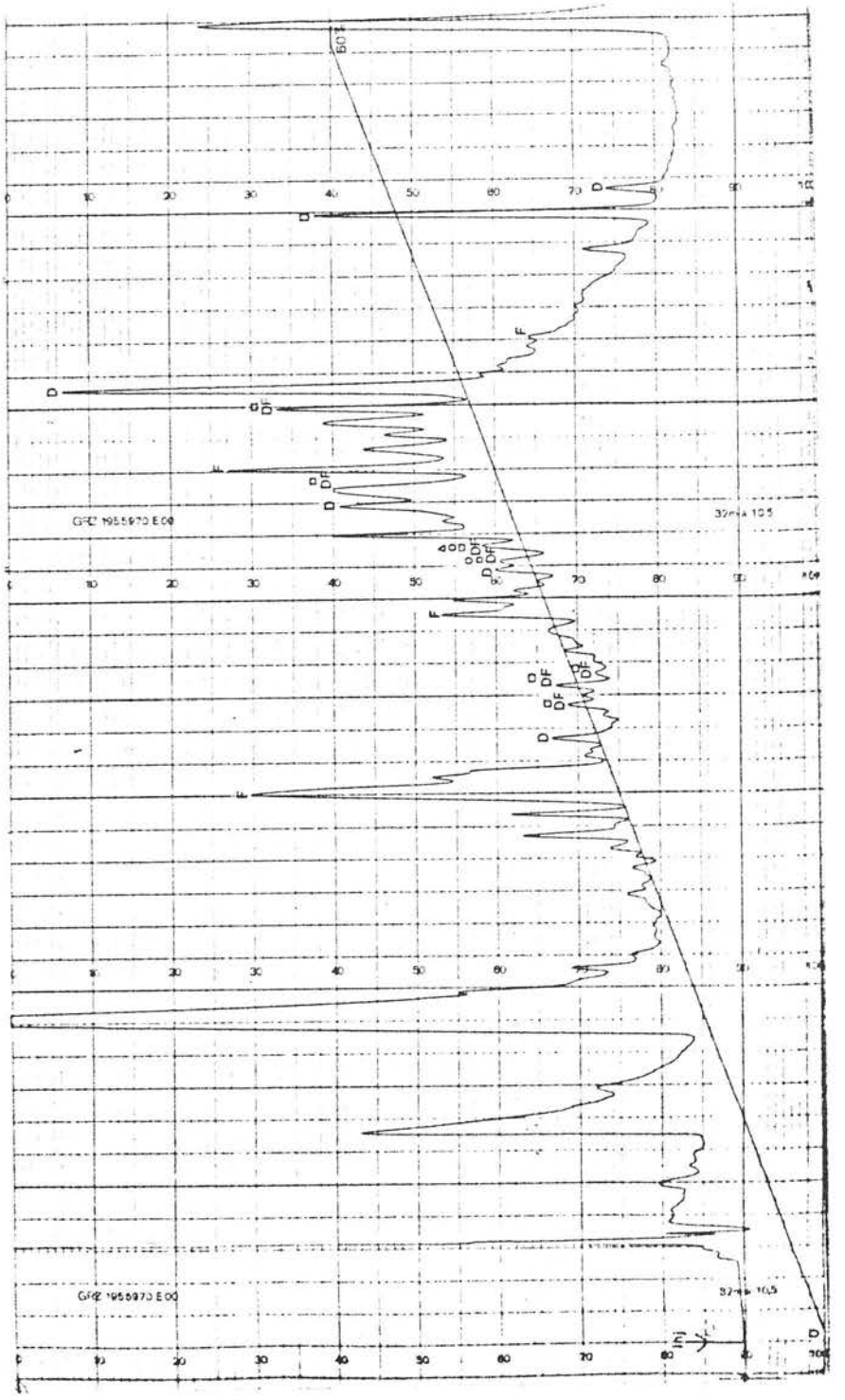

GRZ 1955970 E 00
32mA 10 5
GRZ 1955970 E 00
32mA 10,5
Inj

FIGURE 3.46 Peptide "map" of binder 5D.

A portion of binder 5D (500 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (75 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - See legend to Figure 3.45.

INJ: injection.

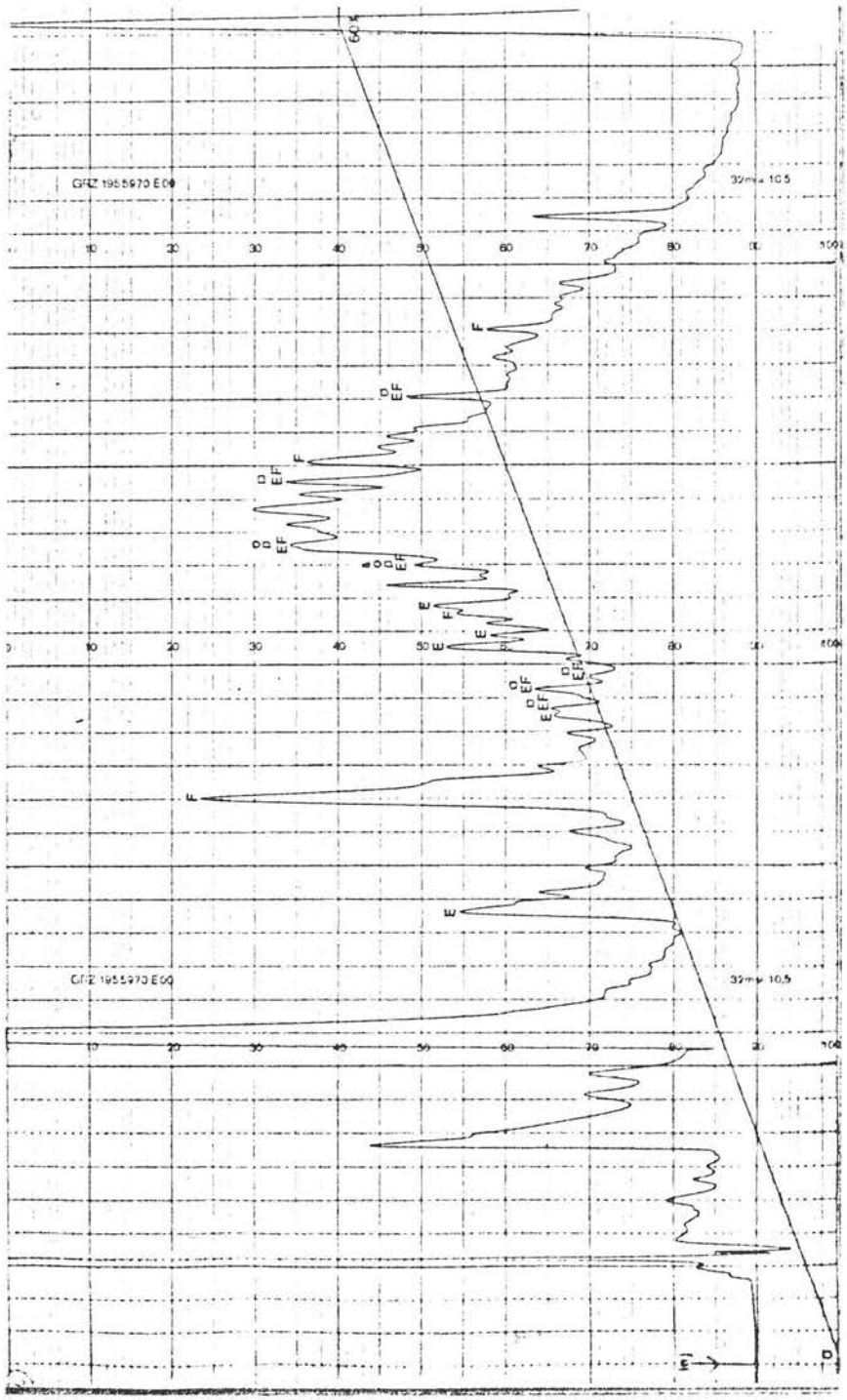

GFZ 1955970 E00
32mV x 10.5
GFZ 1955970 E00
32mV x 10.5

FIGURE 3.47 Peptide "map" of binder 8C.

A portion of binder 8C (500 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (100 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - See legend to Figure 3.45.

INJ: injection.

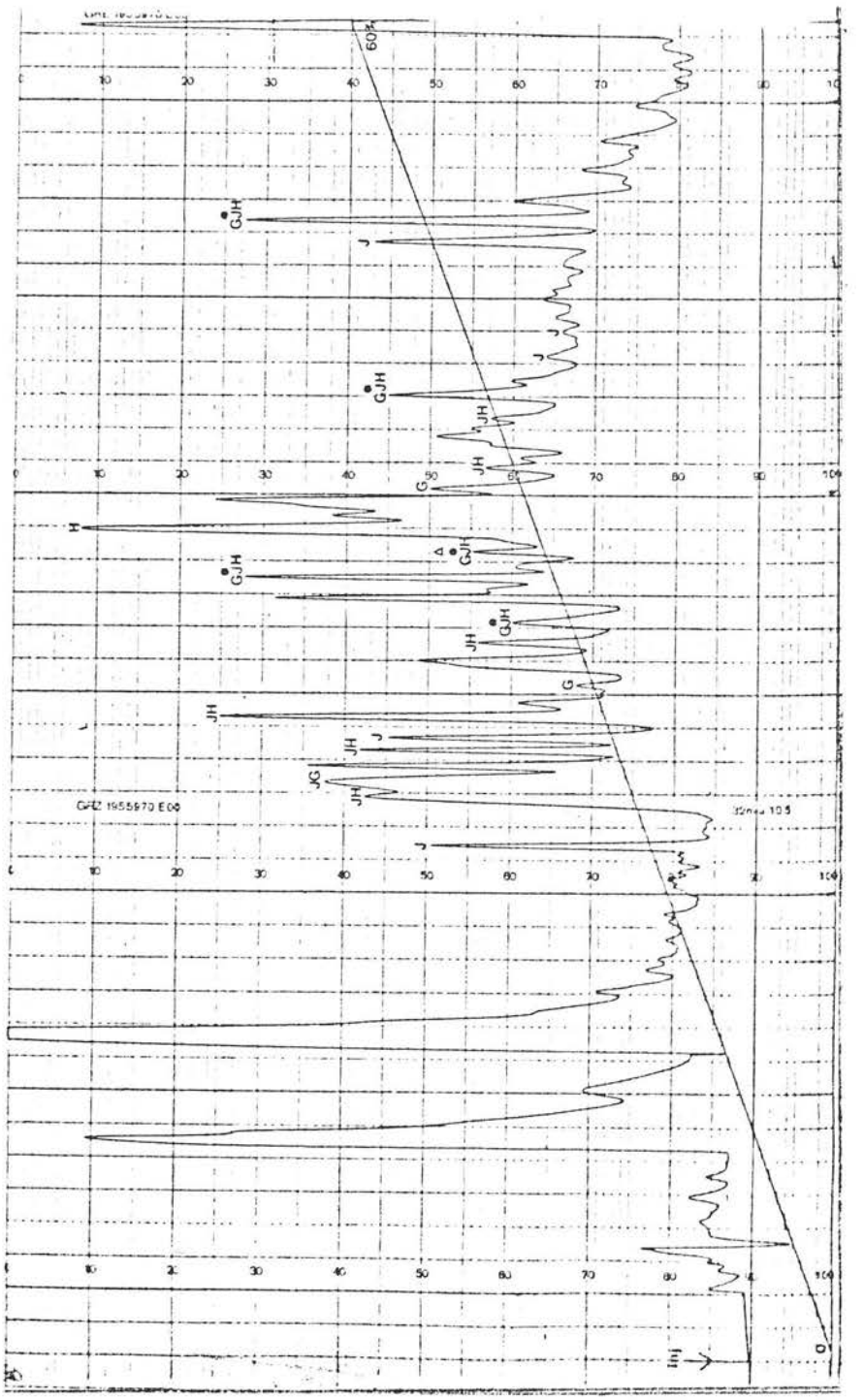

60°
GJH
J
GJH
JH
JH
G
H
GJH
GJH
JH
G
JH
JH
J
JG
JH
J
GHZ 1955970 E06
32mm 10 S

FIGURE 3.48 Peptide "map" of glutathione S-transferase AA

A portion of glutathione S-transferase (500 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (125 µg) were eluted from a reverse-phase HPLC column with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - G: AA-A K: A-D
H: AA-D L: A-F
J: AA-F M: D-F

●: common to glutathione S-transferases AA, A, D and F.

△: common to Y' binders and glutathione S-transferases AA, A, D and F.

INJ: injection.

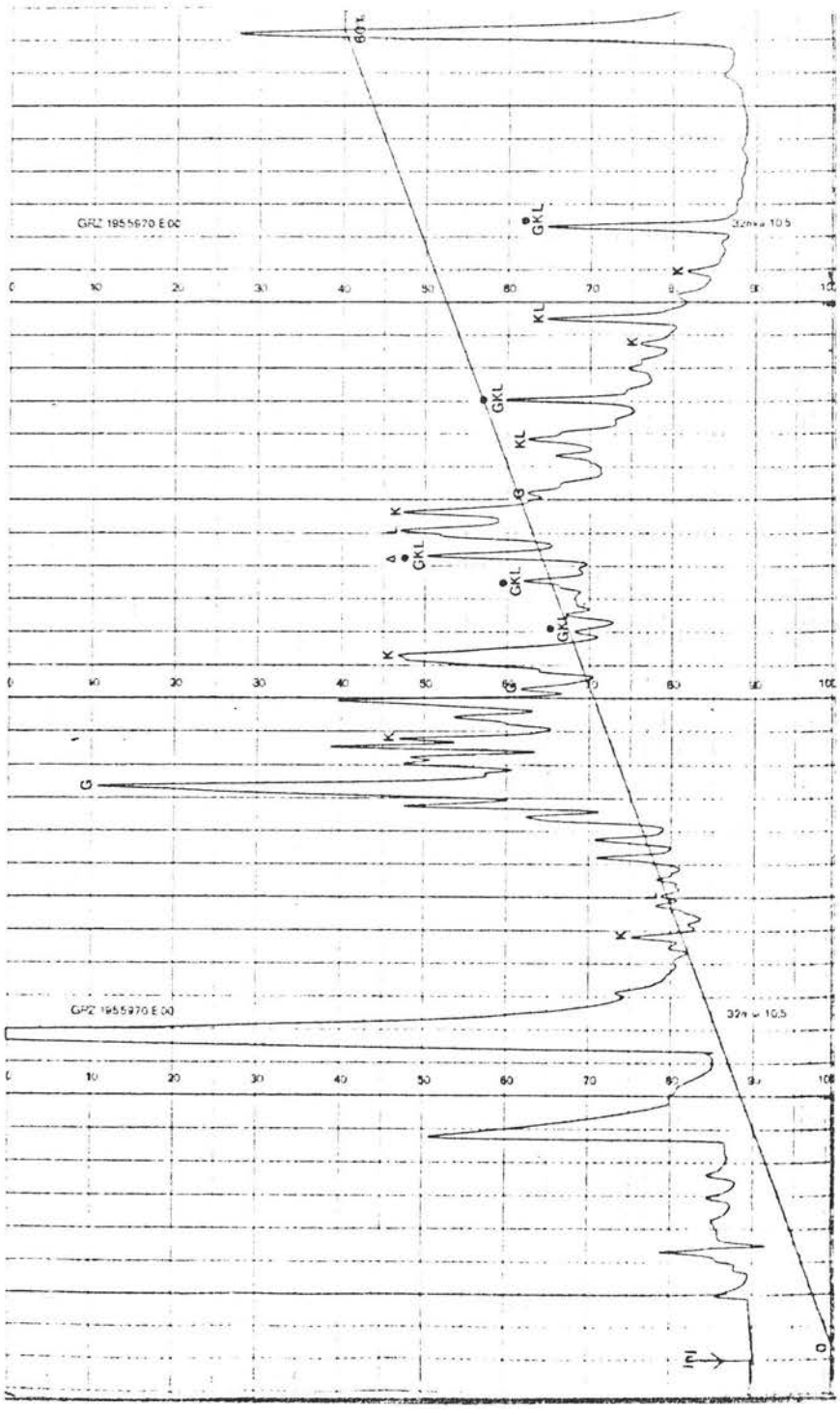

GRZ 1955970 E 00
32n w 10.5
GKL
KL
K
L
G
0
10
20
30
40
50
60
70
80
90
100

FIGURE 3.49 Peptide "map" of glutathione S-transferase A.

A portion of glutathione S-transferase A (500 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (85 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 2 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - See legend to Figure 3.48.

INJ: injection.

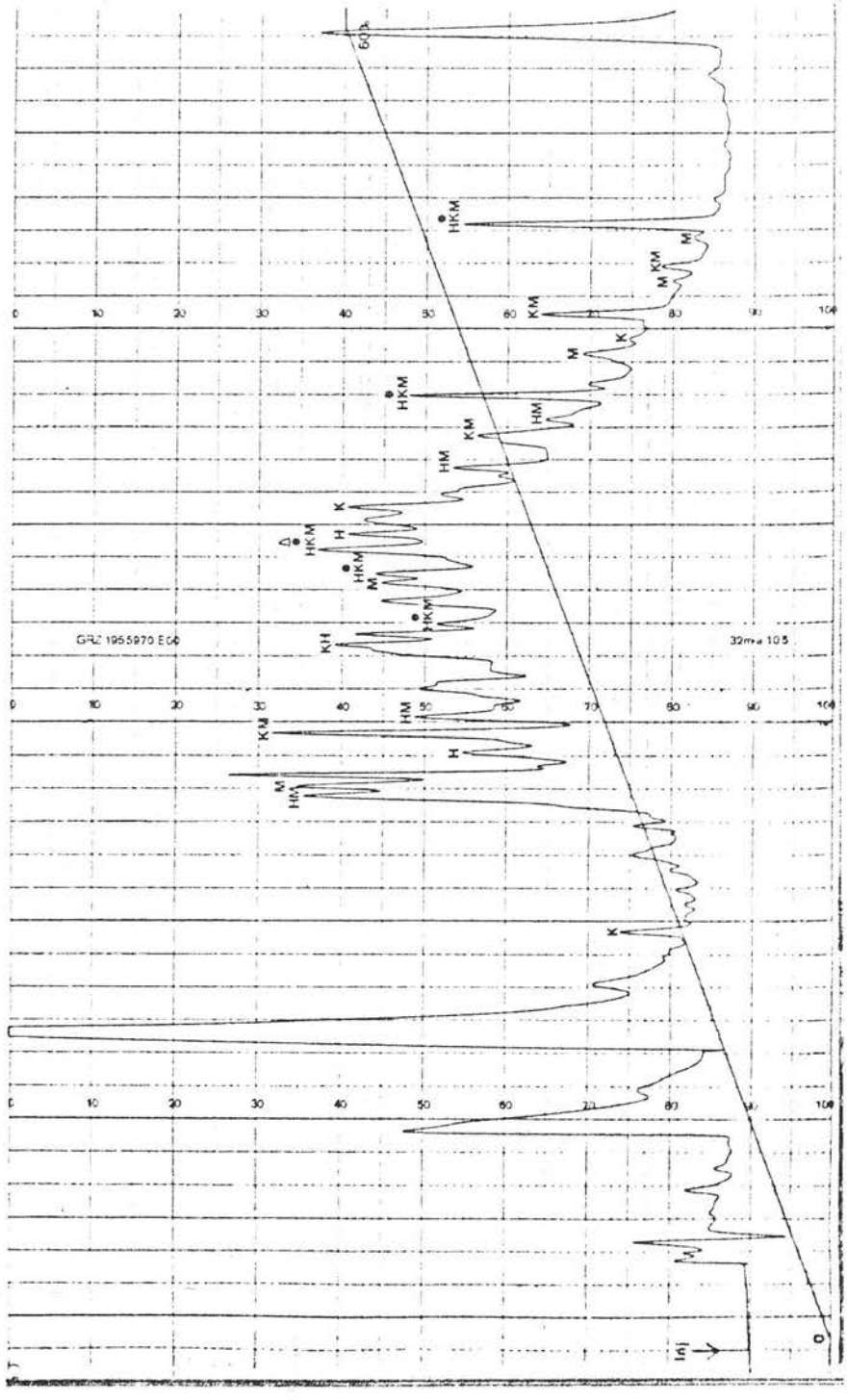

GRZ 1955970 E00
32m•a 10 5
60s
Inj
0

FIGURE 3.50 Peptide "map" of glutathione S-transferase

A portion of glutathione S-transferase (500 μg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (100 μg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - See legend to Figure 3.48.

INJ: injection.

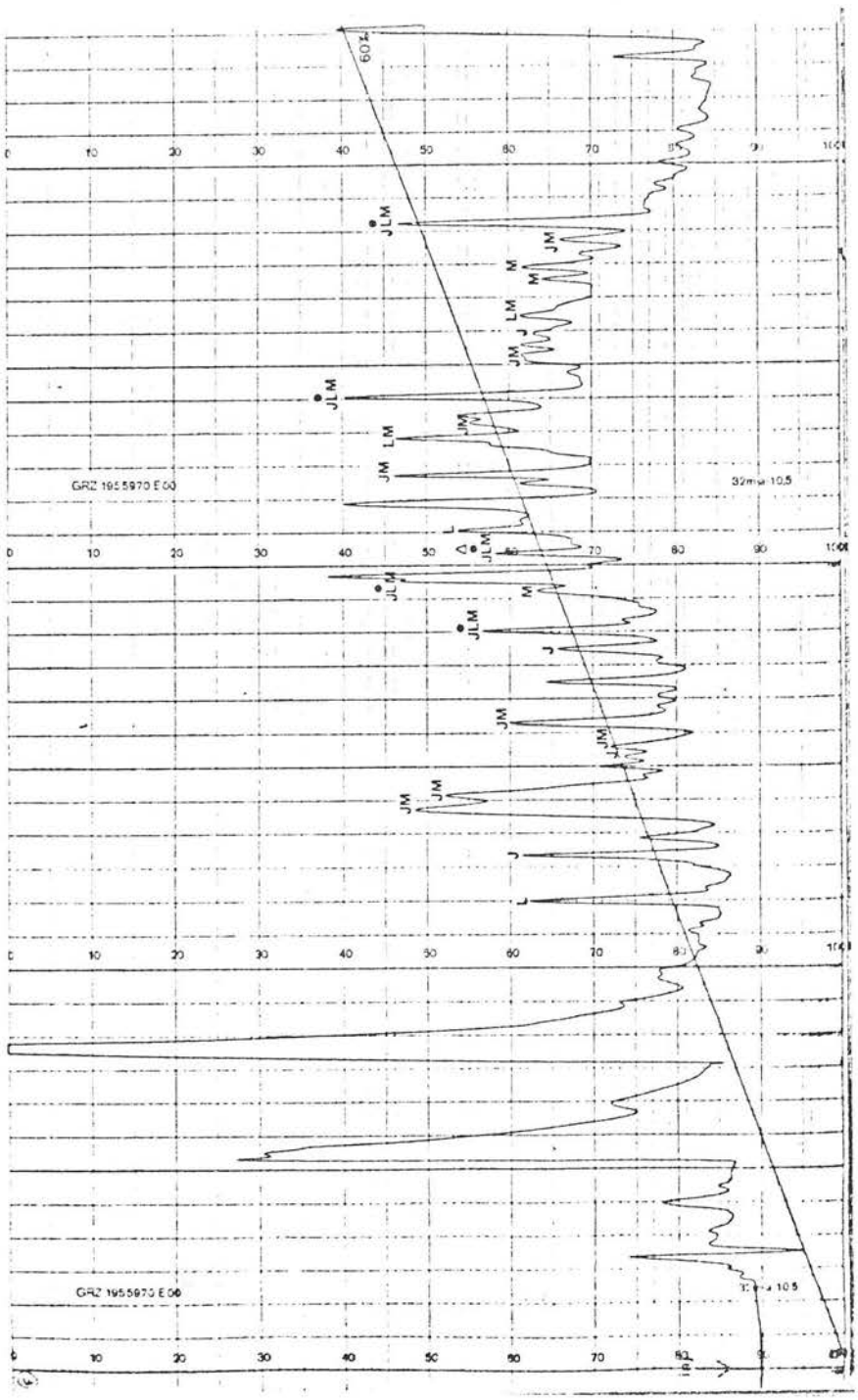

60%
GRZ 1955970 E 00
32m-4 10.5
GRZ 1955970 E 06
32m-4 10.5

FIGURE 3.51 Peptide "map" of glutathione S-transferase F.

A portion of glutathione S-transferase F (500 µg) was oxidised with performic acid and then digested with trypsin. The resulting peptides (100 µg) were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 -60%) of acetonitrile. The flow rate was 1 ml/min and the peptides were detected by their absorbance at 254 nm.

Key to common peaks - See legend to Figure 3.48.

INJ: injection.

Table 3.9 Comparison of peptide "maps" of Y' bile acid-binding proteins

	Comparison of binders	Number of common peptides	Key symbol on peptide "maps"
Group 1	5B - 7F	4	A
	5B - 7F	4	B
	6E - 7F	20	C
	5B-6E-7F	4	*
Group 2	5C - 5D	13	D
	5C - 8C	12	E
	5D - 8C	11	F
	5C-5D-8C	7	□
Group 1 - Group 2		2	O

Refer to Figures 3.42 to 3.47 for peptide "maps" of Y' bile acid-binding proteins.

Table 3.10 Comparison of peptide "maps" of glutathione S-transferases AA, A, D and F

Comparison of binders	Number of common peptides	Key symbol on peptide "maps"
Glutathione S-transferase		
AA-A	8	G
AA-D	12	H
AA-F	17	J
A - D	13	K
A - F	9	L
D - F	18	M
AA-A-D-F	5	●

Refer to Figures 3.48 to 3.51 for peptide "maps" of glutathione S-transferases AA, A, D and F.

Table 3.11 Comparison of peptide "maps" of Y' bile acid-binding proteins and glutathione S-transferases AA, A, D and F

Comparison of binders	Number of common peptides	Key symbol on peptide "maps"
Y' binders		
Group 1 - Group 2	2	0
Glutathione S-transferases		
AA-A-D-F	5	●
Y' and GST	1	△

Refer to Figures 3.42 to 3.51 for peptide "maps" of Y' bile acid-binding proteins and glutathione S-transferases AA, A, D and F.

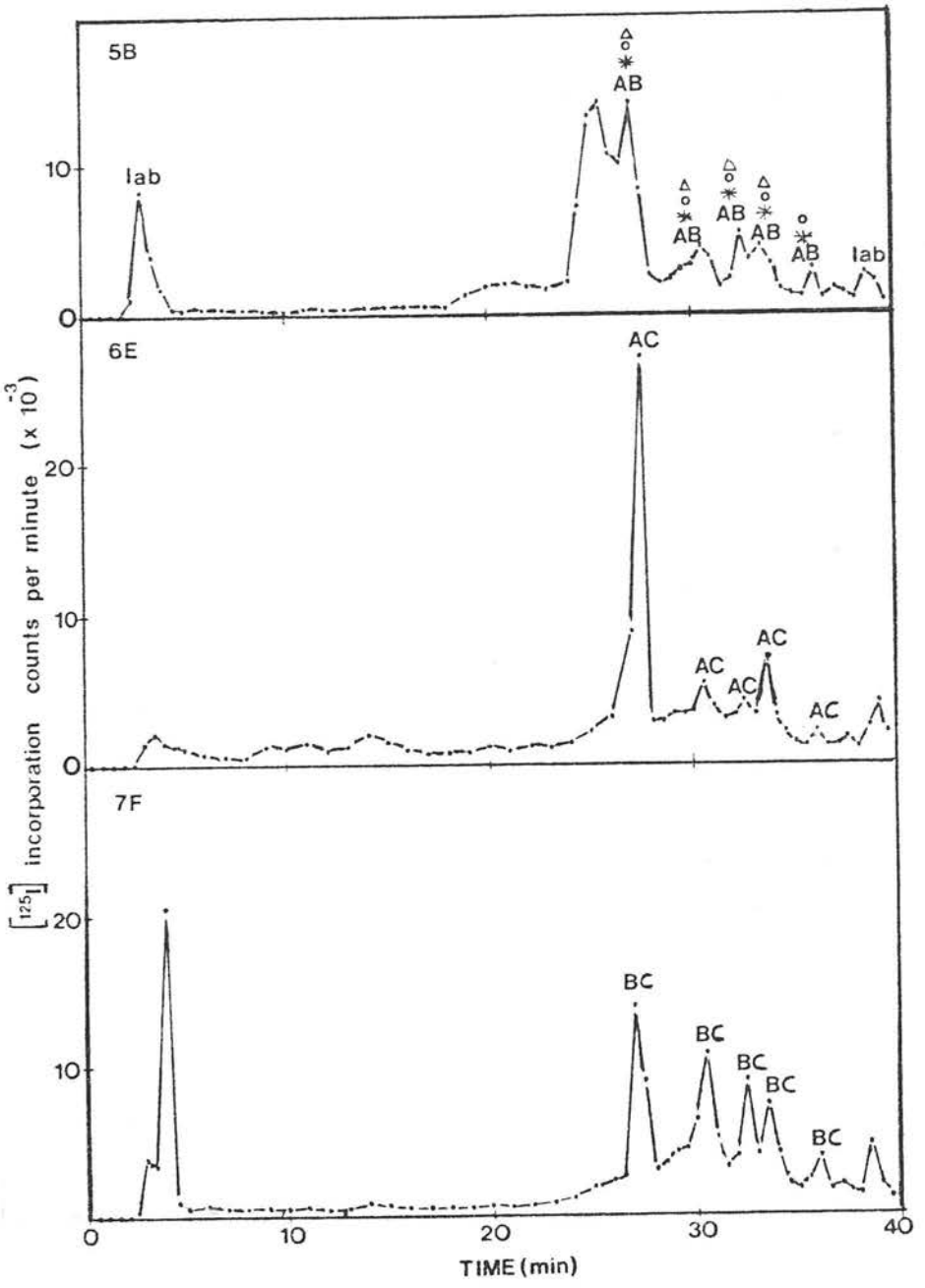

5B
6E
7F
lab
AB
AC
BC
[125I] incorporation counts per minute (x 10-3)
TIME (min)
0
10
20
30
40

FIGURE 3.52 Peptide "maps" of photoaffinity labelled group 1 Y' binders.

Portions of binders 5B (500 μg), 6E (200 μg) and 7F (600 μg) were photoaffinity labelled with [^{125}I]-3βazidocholylhistamine (10 μCi, 5 pmol) for 15 min. The proteins were then oxidised with performic acid and digested with trypsin. The resulting peptides were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and fractions of 0.5 ml were collected and counted for radioactivity. The position of the free, unreacted photoaffinity label is also shown (lab).

Key to common peaks - A: 5B - 6E

B: 5B - 7F

C: 6E - 7F

*: common to group 1 binders

o: common to group 1 and group 2 binders

△: common to Y binders and glutathione S-transferases AA, A, D and F.

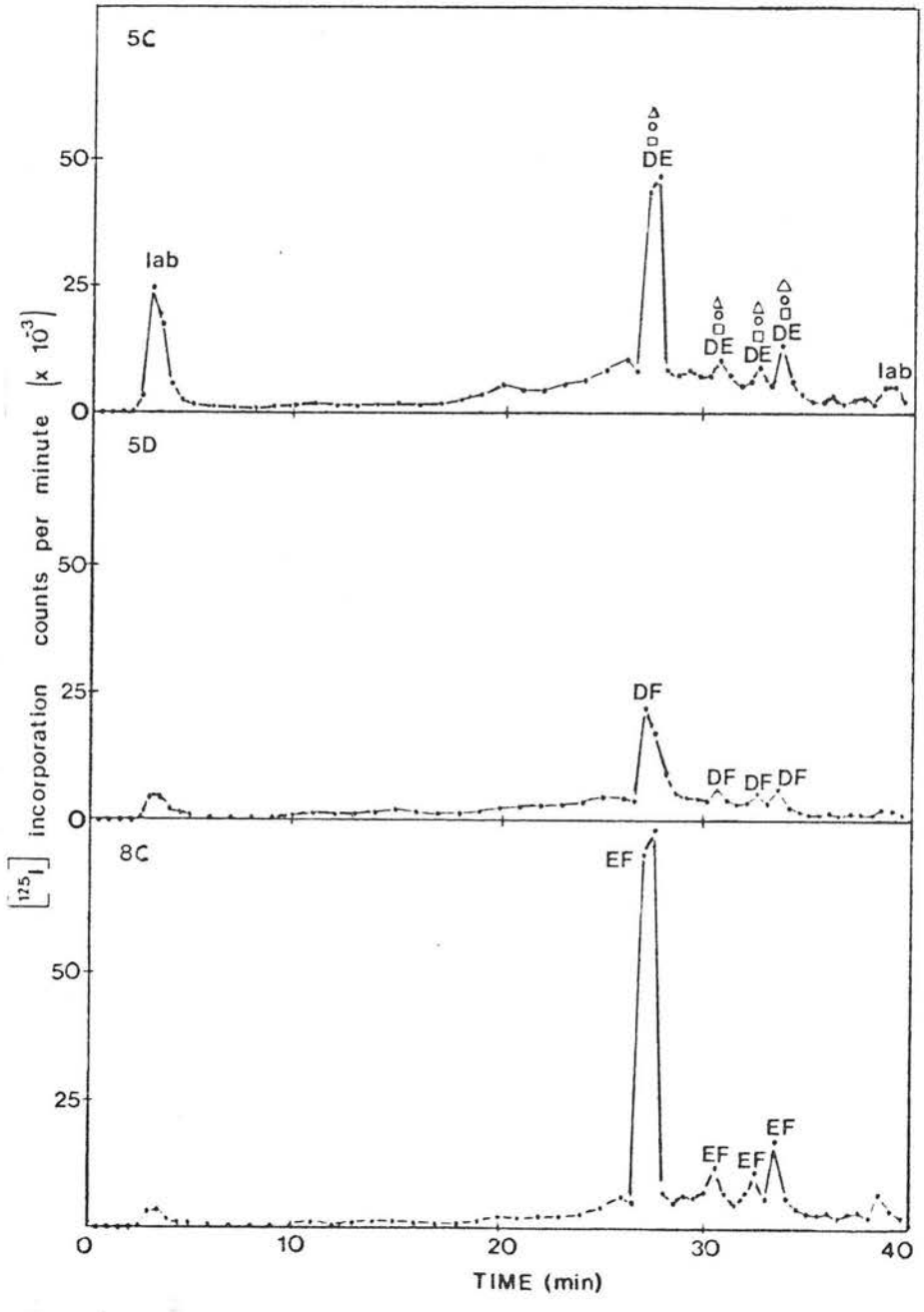

5C
50
25
0
Iab
DE
DE
DE
DE
DE
Iab
5D
50
25
0
DF
DF
DF
DF
8C
50
25
EF
EF
EF
EF
0
10
20
30
40
TIME (min)
[125I] incorporation counts per minute (x 10-3)

FIGURE 3.53 Peptide "maps" of photoaffinity labelled group 2 Y^1 binders.

Portions of binders 5C, 5D and 8C (500 μg) were photoaffinity labelled with [^{125}I]-3βazidocholylhistamine (10 μCi, 5 pmol) for 15 min. The proteins were then oxidised with performic acid and digested with trypsin. The resulting peptides were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and fractions of 0.5 ml were collected and counted for radioactivity. The position of the free, unreacted label is also shown (lab).

Key to common peaks - D: 5C - 5D

E: 5C - 8C

F: 5D - 8C

□: common to group 2 binders

o: common to group 1 and group 2 binders

△: common to Y^1 binders and glutathione S-transferases AA, A, D and F

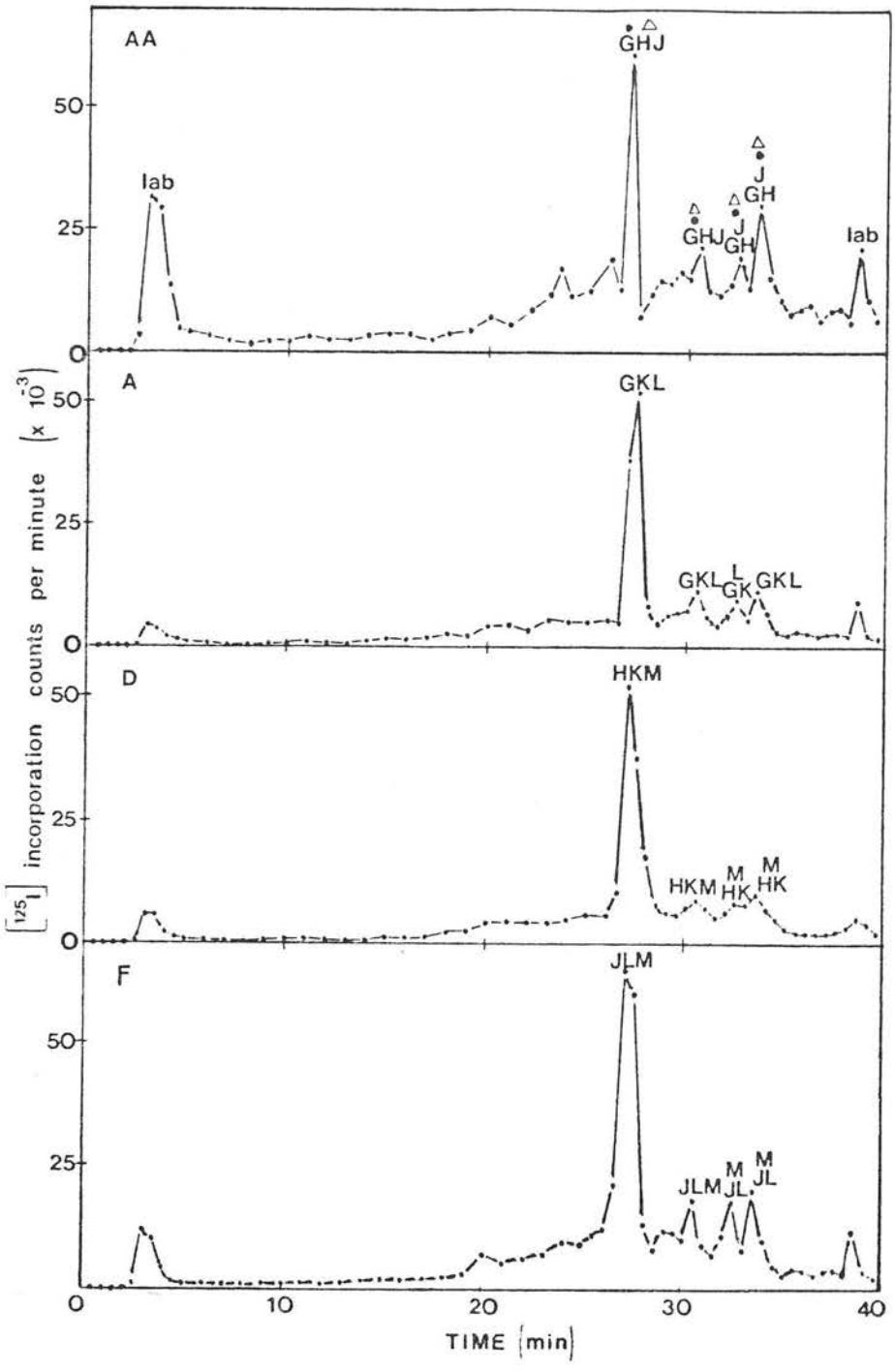

AA
GHJ
Iab
GHJ
J
GH
J
GH
Iab
A
GKL
GKL
L
GK
GKL
D
HKM
HKM
M
HK
M
HK
F
JLM
JLM
M
JL
M
JL
50
25
0
10
20
30
40
TIME (min)
[125I] incorporation counts per minute (x 10-3)

FIGURE 3.54 Peptide "maps" of photoaffinity labelled glutathione S-transferases AA, A, D and F. Portions of GST AA, A, D and F (500 μg each) were photoaffinity labelled with [^{125}I]-3βazidocholylhistamine (10 μCi, 5pmol) for 15 min. The proteins were then oxidised with performic acid and digested with trypsin. The resulting peptides were eluted from a reverse-phase HPLC column (0.39 cm x 30 cm) with a linear gradient (0 - 60%) of acetonitrile. The flow rate was 1 ml/min and fractions of 0.5 ml were collected and counted for radioactivity. The position of the free, unreacted label is also shown (lab).

Key to common peaks - G: AA-A K: A-D
H: AA-D L: A-F
J: AA-F M: D-F

●: common to glutathione S-transferases AA, A, D and F

△: common to Y′ binders and glutathione S-transferases AA, A, D and F.

Table 3.12 Comparison of peptide "maps" of photoaffinity Y' bile acid-binding proteins

	Comparison of binders	Number of common peptides	Key symbol on peptide "maps"
Group 1	5B - 6E	5	A
	5B - 7F	5	B
	6E - 7F	5	C
	5B-6E-7F	5	*
Group 2	5C - 5D	4	D
	5C - 8C	4	E
	5D - 8C	4	F
	5C-5D-8C	4	
Group 1 - Group 2		4	O

Refer to Figures 3.52 and 3.53 for peptide "maps" of photoaffinity labelled Y' bile acid-binding proteins.

TABLE 3.13 Comparison of peptide "maps" of photoaffinity labelled glutathione S-transferases AA, A, D and F

Comparison of binders	Number of common peptides	Key symbol on peptide "maps"
Glutathione S-transferase		
AA-A	4	G
AA-D	4	H
AA-F	4	J
A - D	4	K
A - F	4	L
D - F	4	M
AA-A-D-F	4	●

Refer to Figure 3.54 for peptide "maps" of photoaffinity labelled glutathione S-transferases AA, A, D and F.

Table 3.14 Comparison of peptide "maps" of photoaffinity labelled Y' bile acid-binding proteins and gluthathione S-transferases AA, A, D and F

Comparison of binders	Number of common peptides	Key symbol on peptide "maps"
Y' binders		
Group 1 - Group 2	4	0
Glutathione S-transferases		
AA-A-D-F	4	●
Y' and GST	4	△

Refer to Figures 3.52 to 3.54 for peptide "maps" of photoaffinity labelled Y' bile acid-binding proteins and glutathione S-transferases AA, A, D and F.

Tryptic digestion and analysis on thin-layer chromatography

The Y' bile acid-binding proteins and glutathione S-transferases which had been photoaffinity labelled and then digested with trypsin were also analysed by thin-layer chromatography. The results are shown in Figures 3.55 to 3.60, and Tables 3.15 and 3.16.

Two solvent systems were employed -

A) Chloroform:methanol:30% ammonia

(2:2:1 v/v/v)

B) n-butanol:2-propanol:acetic acid:water:pyridine

(15:10:3:12:10 v/v/v/v/v)

- as recommended for separation of peptides on silica gel (Brown et al., 1975). A portion of unreacted [^{125}I]-3β-azidocholylhistamine was also run on TLC in order to determine the mobility of the free molecule in both solvent systems.

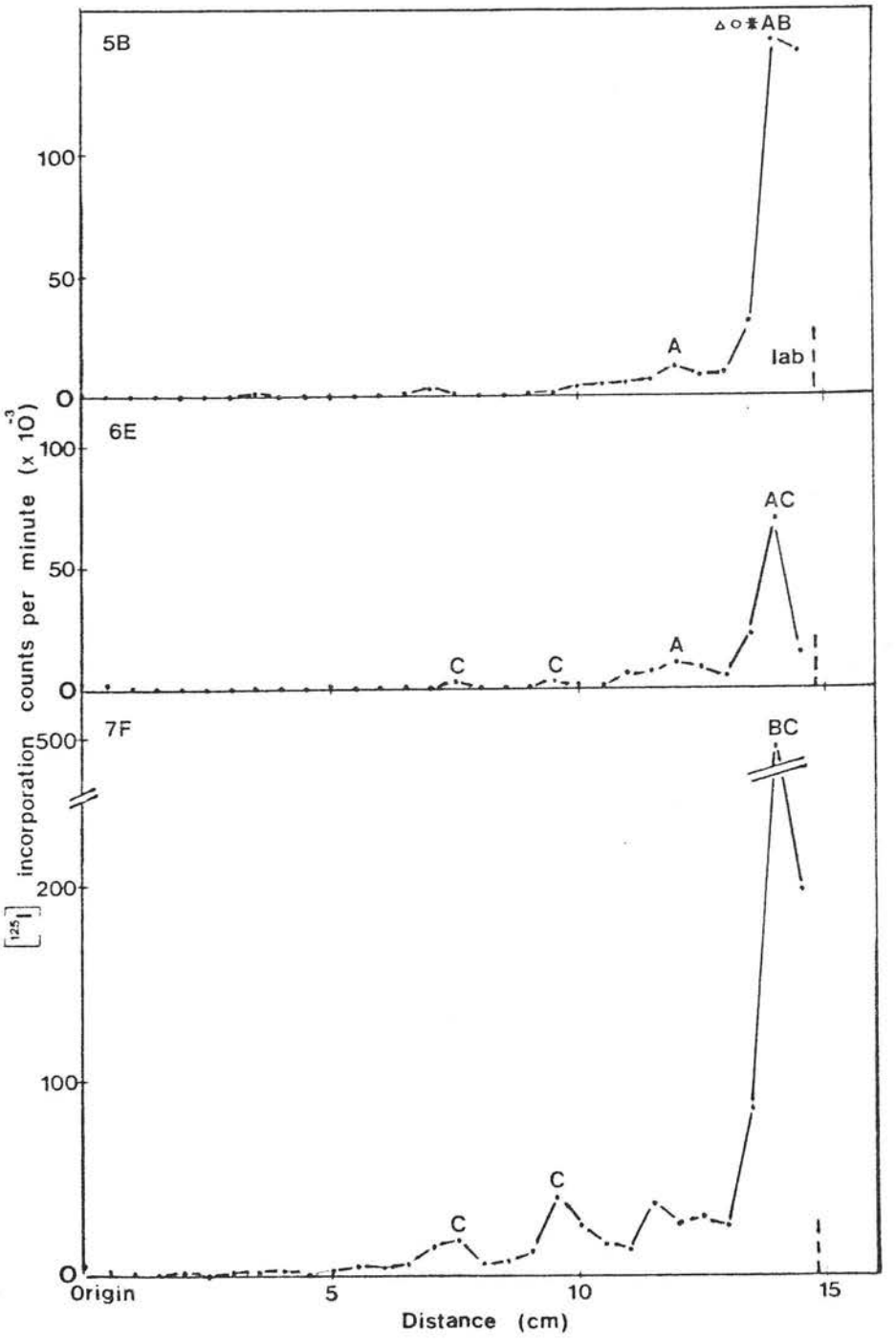

5B
△○*AB
100
50
0
A
lab
6E
100
50
0
AC
C
C
A
7F
500
200
100
0
BC
C
C
Origin
5
10
15
Distance (cm)
[125I] incorporation counts per minute (x 10-3)

FIGURE 3.55 Separation of peptides from the tryptic digestion of photoaffinity labelled group 1 Y' binders by TLC, solvent system A

The mixtures of peptides from the tryptic digests of binders 5B, 6E and 7F (100 µg each) were separated by TLC in solvent system A. The developed plate was divided into 0.5 cm squares, the silica scraped into tubes, and counted for radioactivity. The position of the free, unreacted photoaffinity label is also shown (lab).

Key to common peaks - A: 5B-6E

B: 5B-7F

C: 6E-7F

*: common to group 1 binders

o: common to group 1 and group 2 binders

△: common to Y' binders and glutathione S-transferases AA, A, D and F.

Dotted line indicates position of solvent front.

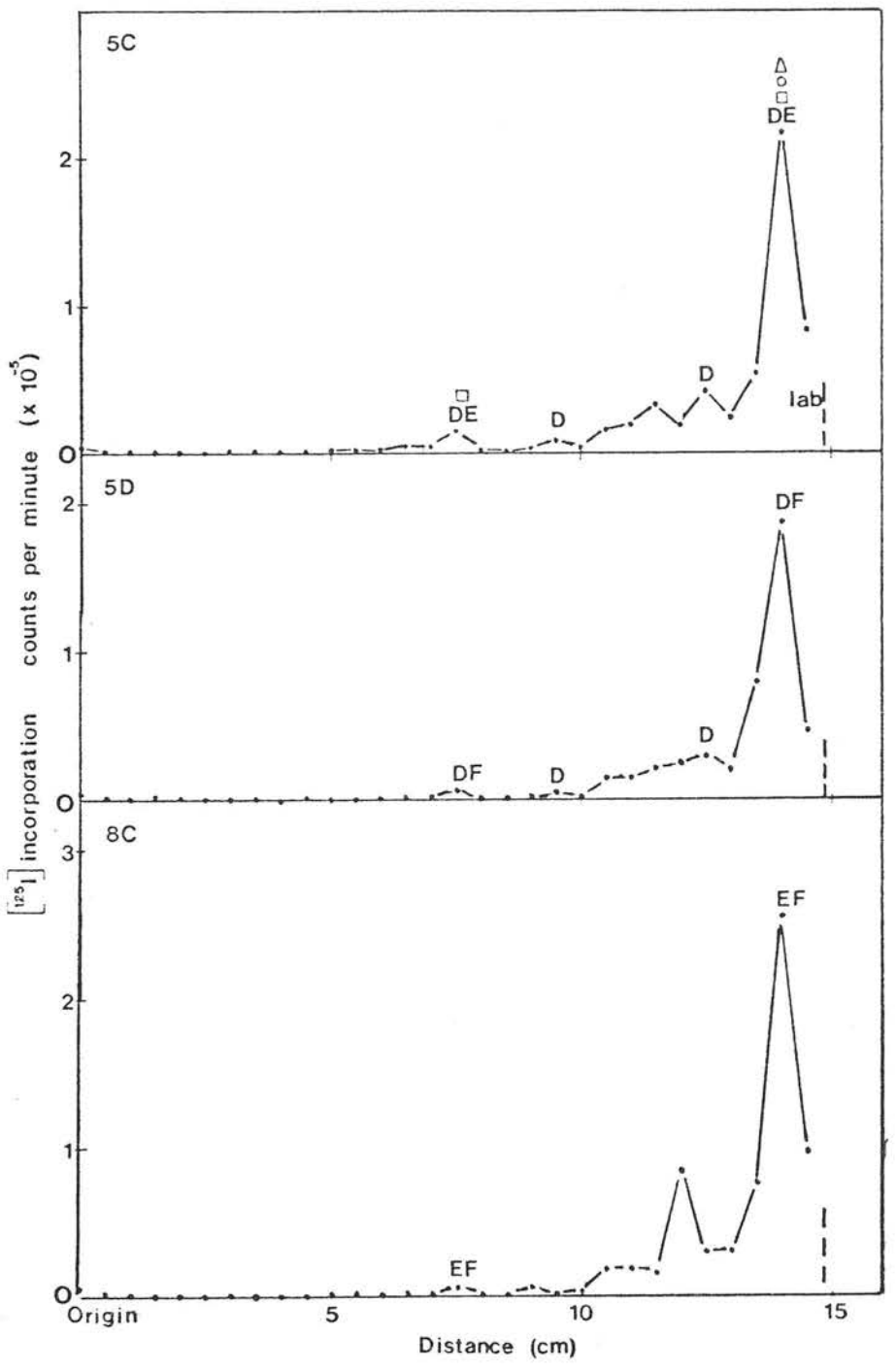

5C
DE
D
lab
5D
DF
D
8C
EF
[125I] incorporation counts per minute (x 10-3)
Origin
5
10
15
Distance (cm)

FIGURE 3.56 Separation of peptides from the tryptic digestion of photoaffinity labelled group 2 Y' binders by TLC, solvent system A.

The mixtures of peptides from the tryptic digests of binders 5C, 5D and 8C (100 µg each) were separated by TLC in solvent system A. The developed plate was divided into 0.5 cm squares, the silica scraped into tubes, and counted for radioactivity. The position of the free, unreacted photoaffinity label is also shown (lab).

Key to common peaks - D: 5C-5D
E: 5C-8C
F: 5D-8C

□: common to group 2 binders

o: common to group 1 and group 2 binders

△: common to Y' binders and glutathione S-transferases AA, A, D and F.

Dotted line indicates position of solvent front.

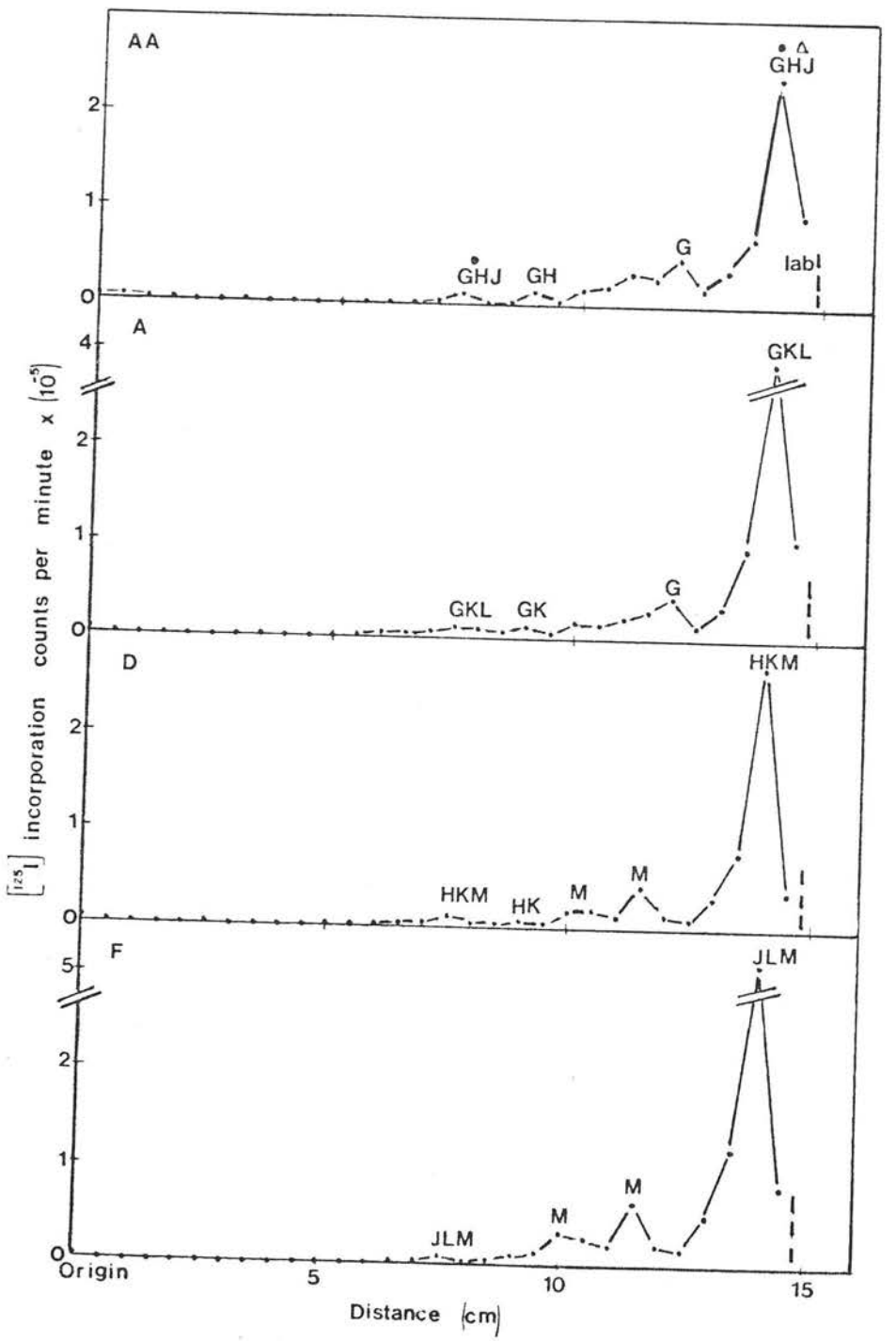

AA
GHJ
GHJ
GH
G
labl
A
GKL
GKL
GK
G
D
HKM
HKM
HK
M
M
F
JLM
JLM
M
M
Origin
5
10
15
Distance (cm)
[125I] incorporation counts per minute x (10-5)

FIGURE 3.57 Separation of peptides from the tryptic digestion of photoaffinity labelled glutathione S-transferases AA, A, D and F by TLC, solvent system A.

The mixtures of peptides from the tryptic digests of glutathione S-transferases AA, A, D and F (100 µg each) were separated by TLC in solvent system A. The developed plate was divided into 0.5 cm squares, the silica scraped into tubes and counted for radioactivity. The position of the free, unreacted photoaffinity label is also shown (lab).

Key to common peaks - G: AA-A K: A-D
H: AA-D L: A-F
J: AA-F M: D-F

●: common to glutathione S-transferases AA, A, D and F

△: common to Y′ binders and glutathione S-transferases AA, A, D and F.

Dotted line indicates position of solvent front.

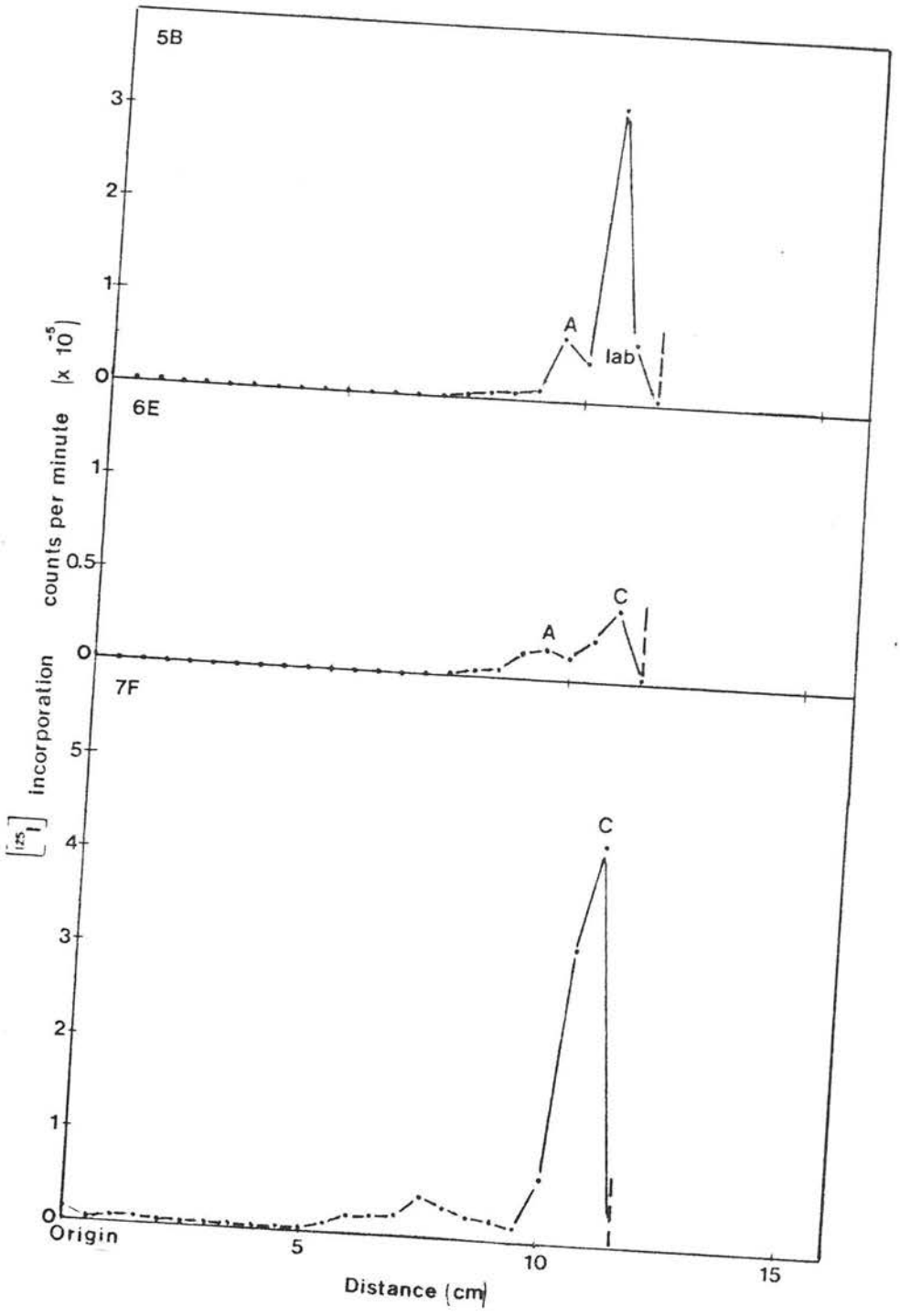

5B
6E
7F
A
lab
C
Origin
5
10
15
Distance (cm)
counts per minute (x 10⁻⁵)
[¹²⁵I] incorporation

FIGURE 3.58 Separation of peptides from the tryptic digestion of photoaffinity labelled group 1 Y' binders by TLC, solvent system B.

The mixtures of peptides from the tryptic digests of binders 5B, 6E and 7F (100 µg each) were separated by TLC in solvent system B. The developed plate was divided into 0.5 cm squares, the silica scraped into tubes, and counted for radioactivity. The position of the free, unreacted photoaffinity label is also shown (lab). A: 5B-6E

B: 5B-7F

C: 6E-7F

*: common to group 1 binders

o: common to group 1 and group 2 binders

△: common to Y' binders and glutathione S-transferases AA, A, D and F.

Dotted line indicates position of solvent front.

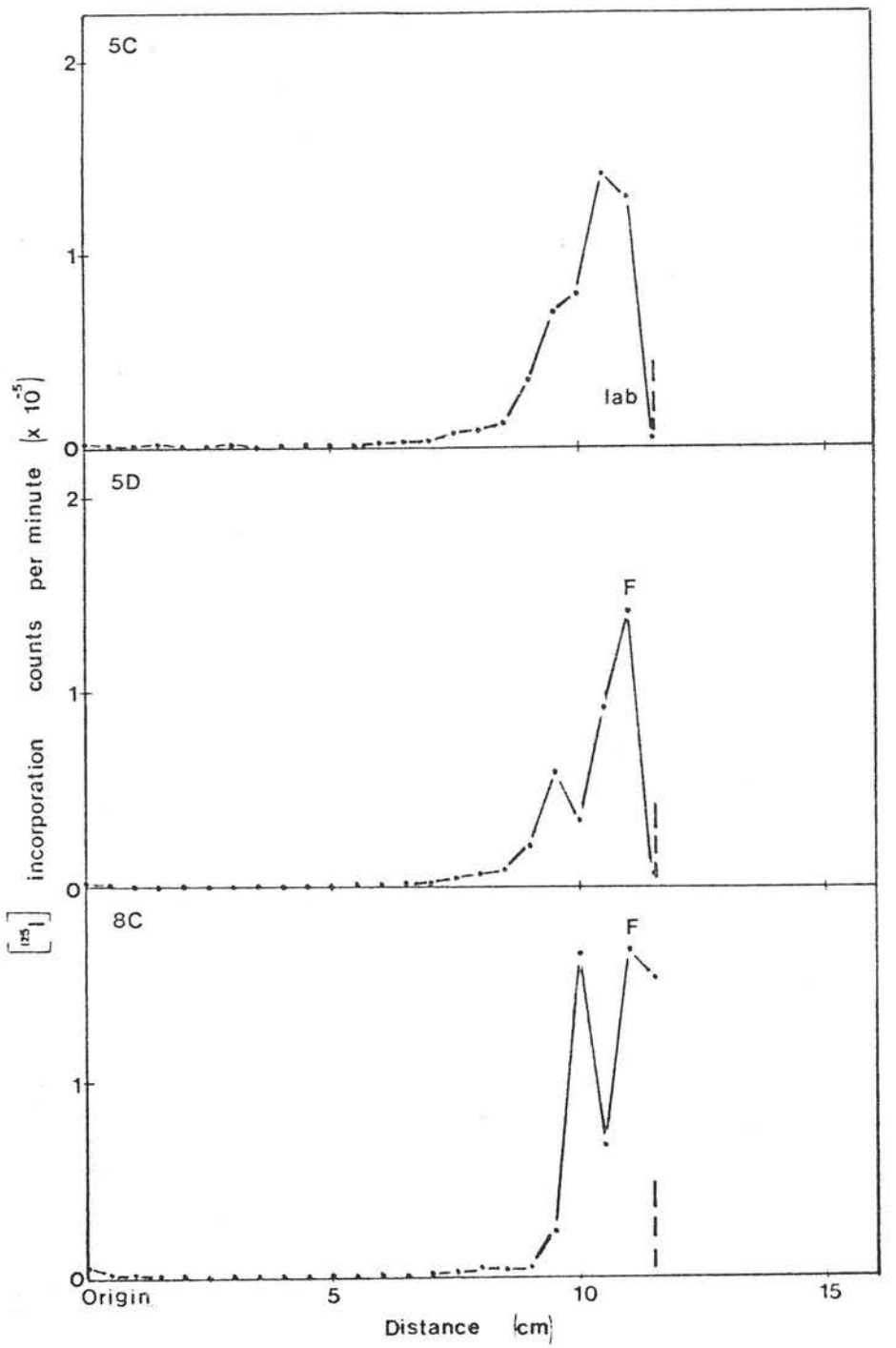

5C
lab
5D
F
8C
F
2
1
0
Origin
5
10
15
Distance (cm)
[125I] incorporation counts per minute (x 10-5)

FIGURE 3.59 Separation of peptides from the tryptic digestion of photoaffinity labelled group 2 Y' binders by TLC, solvent system B.

The mixtures of peptides from the tryptic digests of binders 5C, 5D and 8C (100 µg each) were separated by TLC in solvent system B. The developed plate was divided into 0.5 cm squares, the silica scraped into tubes, and counted for radioactivity. The position of the free, unreacted photoaffinity label is also shown (lab).

Key to common peaks - D: 5C-5D
E: 5C-8C
F: 5D-8C

□: common to group 2 binders

o: common to group 1 and group 2 binders

△: common to Y' binders and glutathione S-transferases AA, A, D and F.

Dotted line indicates position of solvent front.

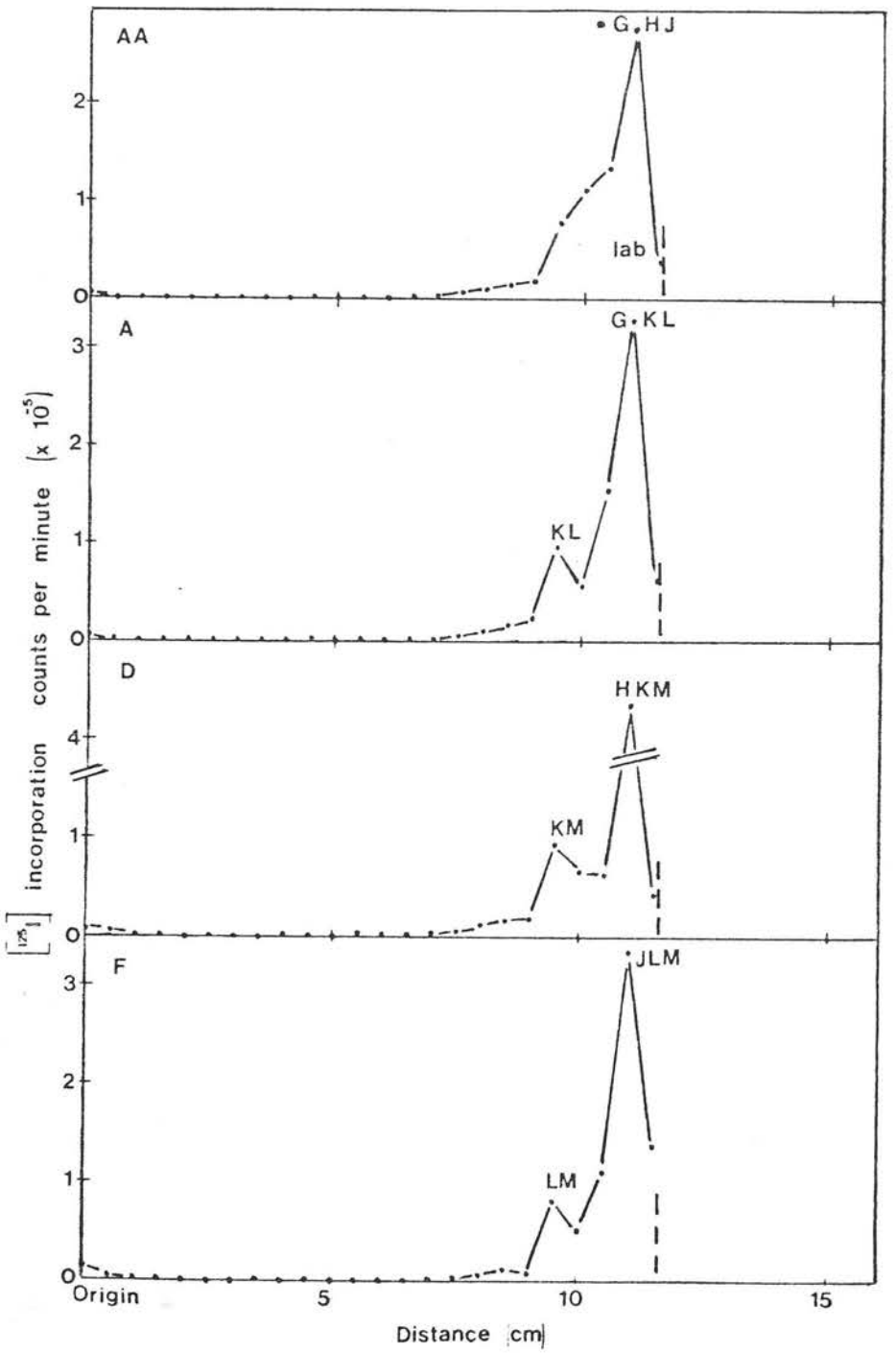

AA
G.HJ
lab
A
G.KL
KL
D
HKM
KM
F
JLM
LM
Origin
5
10
15
Distance (cm)
incorporation counts per minute (x 10⁻⁵)

FIGURE 3.60 Separation of peptides from the tryptic digestion of photoaffinity labelled glutathione S-transferases AA, A, D and F by TLC, solvent system B.

The mixtures of peptides from the tryptic digests of glutathione S-transferases AA, A, D and F (100 µg each) were separated by TLC in solvent system B. The developed plate was divided into 0.5 cm squares, the silica scraped into tubes, and counted for radioactivity. The position of the free, unreacted photoaffinity label is also shown (lab).

Key to common peaks -

G:	AA-A	K:	A-D
H:	AA-D	L:	A-F
J:	AA-F	M:	D-F

●: common to glutathione S-transferases AA, A, D and F

△: common to Y' binders and glutathione S-transferases AA, A, D and F.

Dotted line indicates position of solvent front.

Table 3.15 Comparison of peptide separation, TLC solvent systems A and B, for photoaffinity labelled Y′ bile acid-binding proteins

	Comparison of binders	Number of common peptides (solvent system) A	B	Key symbols on peptide "maps"
Group 1	5B-6E	2	1	A
	5B-7F	1	0	B
	6E-7F	3	1	C
	5B-6E-7F	1	0	*
Group 2	5C-5D	4	0	D
	5C-8C	2	0	E
	5D-8C	2	1	F
	5C-5D-8C	2	0	□
Group 1 - Group 2		1	0	0

Refer to Figures 3.55, 3.56, 3.58 and 3.59 for TLC separation of peptides, in solvent systems A and B, from tryptic digest, Y′ bile acid-binding proteins.

Table 3.16 Comparison of peptide separation, TLC solvent systems A and B, for photoaffinity labelled glutathione S-transferases AA, A, D and F

Comparison of binders	Number of common peptides (solvent system) A	B	Key symbols on peptide "maps"
Glutathione S-transferase			
AA-A	4	1	G
AA-D	3	1	H
AA-F	2	1	J
A-D	3	2	K
A-F	2	2	L
D-F	4	2	M
AA-A-D-F	2	1	●

Refer to Figures 3.57 and 3.60 for TLC separation of peptides, in solvent systems A and B, from tryptic digests of glutathione S-transferases AA, A, D and F.

Table 3.17 Comparison of peptide separation, TLC solvent systems A and B, for photoaffinity labelled $Y^{'}$ bile acid-binding proteins and glutathione S-transferases AA, A, D and F

Comparison of binders	Number of common peptides (solvent system) A	B	Key symbols on peptide "maps"
$Y^{'}$ binders			
Group 1 - Group 2	1	0	0
Glutathione S-transferases			
AA-A-D-F	2	1	●
$Y^{'}$ and GST	1	0	△

Refer to Figures 3.55 to 3.60 for TLC separation of peptides, in solvent systems A and B, from tryptic digests of $Y^{'}$ bile acid-binding proteins and glutathione S-transferases AA, A, D and F.

DISCUSSION

HEPATIC TRANSPORT

Hepatic transport of bile acids and bile salts

Bile acids are the major group of anions transported by the liver - in man and the rat 30 g and 200 mg of (bile acids plus bile salts) are transported each day respectively. The enterohepatic circulation is dependent on this mechanism operating efficiently in order to re-cycle bile acids and bile salts from the intestine back into bile. Transport across the hepatocyte can be broken down into several stages - uptake at the sinusoidal membrane, intracellular transport, and secretion into the canaliculus. Of these, only the uptake process has been well characterised; details of intracellular events are less clear.

Several theories for the transcellular movement of bile acids have been put forward. The diffusion of bile acids across the hepatocyte in free solution, with passive partitioning of the molecules between organelle membranes and the cytosol, has received considerable attention. The rate of transport across the cell would depend on the proportion of bile acid in free solution, and the results of measurement of the diffusion coefficient for various bile acids are apparently compatible with their crossing the liver in free solution.

Another potential route for bile acids is by translocation in membrane-bound vesicles. Several studies have provided evidence in support of this process, with experi-

ments utilising electron microscopic autoradiography to visualise the process at different stages. More recently, however, workers using the microtubule-disrupting agent, colchicine, which interferes with the movement of secretory vesicles within hepatocytes, have shown it to have no effect on the secretion of taurodehydrocholate into bile (Barnwell et al., 1984). These workers suggest that vesicular packaging of bile salts is unlikely to occur during the cellular transit from sinusoidal to canalicular membranes, and that movement of bile salts is probably in free solution in the cytosol.

Several groups of bile acid-binding proteins have been found in the liver; for example the Y fraction (consisting of the glutathione S-transferases), the Z fraction, and more recently the Y' fraction. It is uncertain whether such proteins play an "active" role in bile acid transport, or whether they serve to prevent efflux of bile acids from the hepatocyte as they are concentrated within the cell. Indeed, it is not clear whether the glutathione S-transferases bind bile acids in vivo (Abberger et al., 1983) (see p.258).

Aims of the study

The aims of this thesis were as follows -

1) To synthesize the novel bile acid derivative [^{125}I]-3β -azidocholylhistamine, radioiodinated to a high specific radioactivity.

2)/

2) To use this molecule as a photoaffinity probe in the investigation of bile acid-binding proteins in rat hepatic cytosol.

3) To identify and characterize such proteins from the Y' fraction in particular, and to assess the bile acid-binding capabilities of these proteins.

The results of the studies presented in this thesis will be discussed as follows:-

Photoaffinity labelling;

Purification and characterization of Y' proteins;

Peptide "mapping" of bile acid-binding proteins;

Y' proteins as cytosolic bile acid-binding proteins;

Future experiments.

PHOTOAFFINITY LABELLING

Synthesis of [^{125}I]-3β azidocholylhistamine

Perhaps the most crucial aspect in the synthesis of a photoaffinity label is the design of the molecule. Consideration must be given to the location of the photolabile group within the molecule - ideally it should be positioned such that it does not interfere to any great extent with the binding of the ligand to the binding protein. In addition, photoaffinity probes which are radiolabelled should be constructed such that the radioactive atom is as close as possible to the photolabile group, and not separated from it by any links which may be liable to cleavage during subsequent manipulations. In practice, this latter criterion may be difficult to achieve.

The importance of using reagents, both chemical and radiochemical, of the highest purity, and which are also absolutely dry, cannot be overstressed. Small impurities with a structure similar to the reagent being synthesized could lead to inefficient photoaffinity labelling of the target molecule(s).

The synthesis of [^{125}I]-3βazidocholylhistamine was achieved using cholic acid as starting material (Figure 3.1). The first step involved the selective 3 α--tosylation of this molecule. It was observed on TLC that 3 , 7 di- and 3 , 7 12 -tri-tosylates of cholic acid

also occurred; particularly if the reaction time was extended or an increased concentration of p-toluene sulphonylchloride was used. It was, therefore, necessary to separate the 3α-tosylate from other reaction products by silica gel column chromatography, before the next step in the synthesis.

Addition of the azido group to the 3β position of cholic acid was carried out in the dark and all subsequent manipulations with this molecule were also carried out in dim or red light to avoid destruction of the photolabile group. Addition of histamine to yield 3βazidocholylhistamine gave a stock solution which was stored in ethanol at -20°C. The molecule remained stable in this form for 18 months as demonstrated on TLC.

Iodination of 3βazidocholylhistamine, using the chloramine T reaction, gave a good yield with a high specific radioactivity (approximately 1900 Ci/mmol) after separation on TLC of iodinated from non-iodinated ligand. The iodinated molecule was kept in ethanolic solution at 4°C and used within one month of synthesis. The sensitivity of photoaffinity labelling with this molecule was greater due to the high specific radioactivity, enabling investigation of bile acid-binding proteins which may be present in small quantities in the hepatocyte.

Characterisation of [^{125}I]-3βazidocholylhistamine

The photoaffinity label was characterised extensively

at each stage of the synthesis, using a variety of techniques (Table 3.1). Infra-red spectroscopy served principally to confirm the incorporation of the photolabile azido group into the molecule, (Figures 3.3 to 3.6), with the characteristic peak at approximately 2100 cm^{-1}. Perhaps the most valuable test performed on the molecule, however, was ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The results of these scans confirmed the overall molecular structure of the label, and provided a means of checking the total number of -CH, $-CH_2$ and $-CH_3$ groups present (Figures 3.7 to 3.9).

The hepatic transport of [^{125}I]-3βazidocholylhistamine was assessed by injection of the molecule into the hepatic portal system, both on its own, and with [^{14}C]-taurocholate as a comparison. Subsequent analysis of collected bile (Figures 3.11 and 3.12) showed that not only was the label handled efficiently by rat liver, but that it was transported into bile in essentially the same manner as taurocholate, a naturally-occurring bile salt. Experiments with similar molecules, ^{131}I-, ^{125}I- or ^{123}I-cholylglycylhistamine (Grandjean et al., 1978; Spenny et al., 1978; Jones et al., 1980) have shown that hepatic uptake and excretion of these derivatives is similar to physiological bile salts. Since both these molecules and [^{125}I]-3βazidocholylhistamine are neutral bile acid derivatives, Grandjean et al. (1978) have suggested that the sidechain of a bile acid molecule need not possess an

anionic charge in order to be taken up and excreted by the liver, as had previously been proposed for intestinal transport (Wilson & Treanor, 1975). In addition, the fact that [^{125}I]-3βazidocholylhistamine is transported efficiently by the liver would tend to suggest that alteration of the molecular structure by insertion of an azido group and addition of a histamyl moiety with an [^{125}I] atom has not greatly affected the properties of the derivative as a bile acid. The label was also shown to bind (non-covalently) to components of rat hepatic cytosol by gel filtration (Figures 3.13 and 3.14), and to bovine serum albumin by equilibrium dialysis (Figure 3.10).

Use of [^{125}I]-3βazidocholylhistamine as a photoaffinity label

Photolysis of [^{125}I]-3βazidocholylhistamine was shown to be more successful with ultraviolet light above 300 nm, as judged by the slower breakdown of the derivative (with release of iodinated histamine) on TLC (Figure 3.15) and the greater incorporation of radioactivity into the bovine serum albumin band on SDS/polyacrylamide-gel electrophoresis (Figure 3.17). With ultraviolet light below 300 nm, photolytic destruction of the derivative was rapid, resulting mainly in the release of free iodide, but also, to a lesser extent, in the release of iodinated histamine, due to cleavage of the internal peptide bond between the steroid ring structure and the histamyl moiety. In addition, the breakdown of bovine serum albu-

min on exposure to ultraviolet light as observed on SDS/ polyacrylamide-gel electrophoresis (Figure 3.16) was more marked with ultraviolet light below 300 nm, as might have been expected, since such light contains the biologically-damaging wavelengths at 254 nm and 280 nm.

This last result illustrates an important point in the choice of photolysis conditions for photoaffinity labelling experiments. It has been shown that in some cases the rate of photolysis of the label at 254 nm compares favourably with the rate of destruction of the biological system being labelled (Cooperman & Brunswick, 1973). However, the much more rapid degradation of bovine serum albumin shown here (Figure 3.16) with ultraviolet light below 300 nm demonstrates that this is not always the case. One of the main advantages of arylazides over diazo compounds as photoaffinity labels is the capacity of the former for photolysis at longer wavelengths, for example, above 300 nm, where damage to biological systems is negligible. Indeed, some arylazide compounds have shown higher labelling efficiencies on irradiation with longer wavelengths (Katzenellenbogen, 1974).

With [^{125}I]-3βazidocholylhistamine, ultraviolet light above than 300 nm was chosen, as it has been by other workers, in order to optimise photolysis of the derivative against the potential destruction of the biological system, i.e. the protein(s), being labelled (Kramer *et al.*, 1982, 1983; von Dippe *et al.*, 1983). The fact that

photolysis of the derivative occurred, when the maxima of ultraviolet absorption for [^{125}I]-3βazidocholylhistamine are at 220 nm and 285 nm, is probably due to absorption of light by the molecule at the long wavelength tail of its 285 nm maximum, which is sufficient to stimulate the photolabile azido group. This possibility has been discussed in more detail recently (Nielsen et al., 1983).

Other variables in a photoaffinity labelling experiment, which are in fact inter-related, are the concentration, both of label and target protein(s), and the duration of photolysis. Ideally, photolysis time should be kept as short as possible in order to minimise non-specific labelling; however, this will depend on the half-life of the photoaffinity label when photolysed i.e. the time taken for half the amount of label to react. The half-life of a photoaffinity label varies according to its molecular structure and the substituent groups present within the molecule. Although the half-life of [^{125}I]-3β azidocholylhistamine was not determined owing to the small amounts of material synthesized, it should be approximately the same as a similar derivative, 3βazidocholic acid, used by Kurz and Gerok (1983) and having a half-life of 18.5 min.

The concentration of label used in an experiment is important. To maximise labelling specificity (at the expense, however, of a high extent of site labelling), a

low label concentration is used with respect to the protein concentration. However, in order to achieve a useful level of site labelling, it is then necessary that the label has a very high specific radioactivity; [^{125}I]-3β azidocholylhistamine, for example, has a specific radioactivity of approximately 1900 Ci/mmol.

With many photoaffinity labels, as is the case with [^{125}I]-3βazidocholylhistamine, they must of necessity be added to the reaction system in organic solvent owing to their relative insolubility in water. The amount of organic solvent used should be very small, owing to the possibility of deleterious effects on the biological components of the reaction system. In this study, the label was dissolved in ethanol; the volume of ethanol added with the label to the reaction system was less than 1% of the total volume of the system. In these studies, a ratio of protein:label of approximately 8000:1 (w/w) was employed.

It is also imperative that the photoaffinity label be fully equilibrated with the biological preparation, i.e. protein(s), prior to photolysis. This was achieved in the experiments reported here by mixing the label and protein(s) together in the absence of light at 25^{o}C for 30 min. Longer pre-equilibration times did not increase the extent of covalent labelling of the protein(s).

Initial experiments using [^{125}I]-3βazidocholylhistamine to photoaffinity label bovine serum albumin (Figure 3.17) showed that, with a protein concentration of 10 mg/ml

(label 0.5 pmol, 1 μCi/ml), covalent incorporation of label into the protein was still increasing after a photolysis time of 60 min. However, a later study with one of the putative bile acid-binding proteins from the Y' fraction (Peak 5B), at a lower concentration of 5 μg/ml (label 0.15 pmol, 0.3 μCi/ml), demonstrated that the covalent incorporation of [^{125}I]-3β-azidocholylhistamine reached a maximum between photolysis times of 10 and 20 min (Figure 3.39). Thus, the latter of these two methods was adopted for photoaffinity labelling experiments, having not only the advantage of using less protein, but also being quicker to perform. These results illustrate the screening effects present in a biological preparation, owing to the absorption of ultraviolet light by the protein(s). The half-life of a photoaffinity label can thus be increased not only on binding to the protein, but also in preparations where the protein concentration is high, such as discussed earlier.

Analysis of photoaffinity labelling

Various methods were employed to analyse the photoaffinity labelled rat cytosolic proteins, both as a complex mixture and as pure, individual proteins. In initial experiments with bovine serum albumin, whole rat hepatic cytosol and the glutathione S-transferase fraction, samples were taken at various times during the photolysis and analysed by SDS/polyacrylamide-gel electrophoresis (Figures

3.17, 3.19 and 3.21). This method involved diagrammatically reproducing the polypeptide band patterns from the gels, cutting the gel tracks into slices and counting each slice for ^{125}I. This approach had significant disadvantages. Apart from being time-consuming, potential errors could arise as a result of mis-matching of polypeptides on the gel and the radioactive counts obtained from the gel slices. Further, the method of counting the gel slices themselves will possess an intrinsic error.

In addition, the final, photolysed reaction mixture was analysed as a whole by gel filtration (Figures 3.18 and 3.20) for the bovine serum albumin and whole rat hepatic cytosol experiments. This system was adequate to remove non-covalently bound label from the proteins as evidenced by the appearance of free label in the salt volume of the gel filtration columns. However, large amounts of photolysed proteins were required to use this method.

Alternatively, when the bile acid-binding activity of the partially purified cytosolic proteins was examined (e.g. the Y' peaks from DEAE Sephadex), the reaction mixture obtained after photolysis was precipitated with 10% TCA and the resulting precipitate analysed by SDS/polyacrylamide-gel electrophoresis. However, since in the pellet even after extensive washing non-covalently bound label still remained, it was found necessary to analyse the TCA precipitated-proteins by gel filtration under

denaturing conditions (Table 3.7). The use of small G-25 Sephadex columns (1 cm x 20 cm), equilibrated and eluted with buffer containing 1% SDS, proved sufficient to remove all non-covalently bound label from the photolysed proteins. This was, therefore, found to be the most satisfactory method for quantifying the amount of bile acid incorporated following photolysis. Small amounts of protein (50 µg) could be used with this method, thus enabling each peak from the chromatofocusing of the Y' fraction to be analysed using the photoaffinity label (Table 3.6). Pure glutathione S-transferases AA (YcYc protein), A (Yb_1Yb_1 protein), D (Yb_2Yb_2 protein) and F (YaYa protein) were also analysed in this way, in order to give a comparison between photoaffinity labelled Y and Y' proteins in terms of covalent incorporation of radioactivity.

PURIFICATION AND CHARACTERIZATION OF Y' PROTEINS

Purification of Y' proteins

The major bile acid- and organic anion-binding components of rat hepatic cytosol have been considered to be the glutathione S-transferases (Strange et al., 1977a, b; Hayes et al., 1981). This family of enzymes elutes as a single peak (the Y fraction) on Sephadex G-100 chromatography, with a molecular mass range of approximately 50 000 - 60 000 Da. Recently. however, the bile acid-binding and the organic anion-binding within the Y' fraction have both been split into two distinct peaks of activity (Sugiyama et al., 1982, 1983). The glutathione S-

transferases were reported to be responsible for one peak of bile acid-binding and organic anion-binding activity whilst the other peak of binding was attributed to a newly discovered fraction, previously hidden within the Y peak, and termed the Y' fraction.

Sugiyama et al. (1982) reported that the Y' fraction contains proteins with an affinity for organic anions comparable to the glutathione S-transferases. In addition, proteins from this fraction, along with glutathione S-transferase B, were suggested to be the major hepatic cytosol bile acid-binding proteins in the rat. Two bile acid-binding proteins, designated binders I and II, have been purified from the Y' fraction and partially characterised (Sugiyama et al., 1983). The purification procedure for these proteins is shown in Table 4.1, and Table 4.2 shows the purification scheme for Y' proteins presented in this thesis. (This is an abridged version of Table 2.3).*

The major difference between the two purification procedures lies in the number of proteins resolved. The introduction of an ion-exchange step between the gel filtration and chromatofocusing stages resulted in greater resolution of the Y' fraction. The DEAE Sephadex step was particularly useful since it enabled a significant proportion of the non-binding proteins to be discarded prior to chromatofocusing. This, therefore, reduced the

*NOTE: The possibility exists, however, that some of these proteins may be derived from plasma - see p.123.

Table 4.1 Purification scheme bile acid binders I and II (Sugiyama et al., 1983)

1) Rat liver 100,000 g supernatant, dialysed and eluted from Sephadex G-100 fine. Fractions with glutathione S-transferase activity pooled and concentrated.

2) Concentrated material eluted from Sephadex G-75 superfine. Fractions with Y′ proteins on SDS/PAGE pooled and concentrated.

3) Concentrated material eluted from Sephadex G-75 superfine. Y′ peak pooled and dialysed.

4) Y′ proteins chromatofocused with linear pH gradient from 7.4 to 4. Binder I and Binder II resolved.

5) Binder I eluted from hydroxyapatite, with linear phosphate gradient, 0-150 mM. Seven peaks resoloved, major bile acid-binding activity, peak 7. Binder I has molecular mass 33 000 Da, pI 5.6.

6) Binder II eluted from hydroxyapatite, linear phosphate gradient, 0-150 mM. Five peaks resolved, major bile acid-binding activity, peak 4. Binder II has molecular mass 33 000 Da, pI 5.5.

Table 4.2 Purification scheme for Y' proteins

1) Rat liver 100,000 g supernatant, dialysed and eluted from Sephadex G-100 fine. Fractions with glutathione S-transferase activity pooled and concentrated.

2) Concentrated material eluted from Sephadex G-75 superfine. Fractions with Y' proteins on SDS/PAGE pooled and concentrated.

3) Concentrated material eluted from Sephadex G-75 superfine. Y' peak pooled and dialysed.

3a) Y' peak eluted from DEAE Sephadex with a linear 0-200 mM NaCl gradient, then 500 mM and 1 M NaCl steps. Twelve peaks resolved.

3b) DEAE-Y' peaks photoaffinity labelled. Peaks 5, 6, 7 and 8 positive results.

4) Peaks 5, 6, 7 and 8 chromatofocused separately with linear pH gradients from 6.4 to 4.

PEAK 5	PEAK 6	PEAK 7	PEAK 8
Seven peaks resolved	Six peaks resolved;	Seven peaks resolved;	Five peaks resolved;
3 proteins +ve with photoaffinity labelling -	1 protein +ve with photoaffinity labelling -	1 protein +ve with photoaffinity labelling -	1 protein +ve with photoaffinity labelling -
5B: 19 600 Da, pI 5.75	6E: 15 600 Da, pI 5.2	7F: 14 900 Da, pI 4.85	8C: 33 000 Da, pI 4.65
5C: 36 200 Da, pI 5.65			
5D: 36 200 Da, pI 5.45			

5) Further purification on hydroxyapatite or re-chromatofocusing as necessary.

risk of the chromatofocusing column being overloaded. [^{125}I]-3β azidocholylhistamine was used as a means of assessing the bile acid-binding potential of the Y' proteins both at the ion-exchange and chromatofocusing stages, and thus assisted in the elucidation of the proteins of interest from what was a highly complex mixture of proteins.

The twelve peaks resolved after ion-exchange were photoaffinity labelled and analysed by two different methods (Figure 3.38 and Table 3.8). Both sets of results showed that peaks 5, 6, 7 and 8 gave a higher incorporation of radioactivity and thus contained proteins with significant bile acid-binding activity. Peak 1, although not as great as the other four, showed an increased incorporation of radioactivity above background. The organic anion-binding protein, D_V (Sugiyama et al., 1982), was shown to be present in this peak on the basis of elution position from DEAE Sephadex and mobility on SDS/polyacrylamide-gel electrophoresis. With electrophoresis analysis (Figure 3.38) peak 8 showed the highest degree of labelling, followed by peaks, 6, 7 and 5. However, using the TCA precipitation/denaturing gel filtration method (Table 3.8), peak 5 had the greatest incorporation of label, followed by 6, 7 and 8. This latter situation probably more accurately reflects the ultimate distribution of putative bile acid-binding proteins in the Y' fraction (Table 3.6), and thus the denaturing gel filtration method

was chosen in preference to electrophoretic separation as the method of analysis of photoaffinity labelling.

The subsequent application of chromatofocusing to the peaks of interest (i.e. 5, 6, 7 and 8) from ion-exchange was successful in obtaining purified, homogeneous proteins. Using the denaturing gel filtration method as the means of analysis, all the peaks from the chromatofocusing stage were photoaffinity labelled (Table 3.6). Those proteins designated bile acid-binders have either a high specific ($\geqslant 1 \times 10^5$ counts per minute per 50 μg) and/or high total ($\geqslant 1 \times 10^6$ counts per minute) incorporation of radioactivity. Other proteins also show covalent incorporation of the label, although in most cases this amounts to less than 10% of the lowest total incorporation of radioactivity in the bile acid-binding proteins. This is probably due to non-specific or "background" labelling.

Identity of Y' proteins

As already mentioned, the purification scheme described here (Table 4.2) results in the resolution of a greater number of proteins from the Y' fraction than found by Sugiyama *et al.* (1983) (Table 4.1).

The proteins described for the first time in this thesis are listed in Table 4.3, along with the glutathione S-transferases studied as part of this work.

The group 1 Y' proteins - binders 5B, 6E and 7F - are entirely new, hitherto undescribed, proteins from rat hepatic cytosol which possess bile acid-binding activity.

Table 4.3 Bile acid-binding proteins from the Y' and Y fractions.

Bile Acid-binding protein	Number of Subunits	Subunit Mol. Mass (Da)	Native Mol.Mass (Da)	pH from Chromato-focusing	Specific Incorporation [^{125}I]CPM(x10^{-3})/50 μg	Total Incorporation [^{125}I]CPM(x10^{-6})
[1]5B)	2	19 600	39 200	5.75	113	1.8
6E) Group 1	2	15 600	31 200	5 2	116	1.7
7F)	2	14 900	29 800	4.85	99	1
5C)	1	36 200	36 200	5.65	20	1
5D) Group 2	1	36 200	36 200	5.45	23	1
8C)	1	33 000	33 000	4.65	31	2.8
[2]GST AA	2	28 500	57 000	9.8	160	-
GST A	2	27 000	54 000	8.8	119	-
GST D	2	27 000	54 000	6.9	267	-
GST F	2	25 500	51 000	9.6	186	-

NOTE: GST = glutathione S-transferase; [1] C J Henderson, thesis; [2] Mannervik (1984) and C J Henderson, thesis.

In contrast to previously reported bile acid-binders from the Y' fraction (Sugiyama et al., 1983), which are monomeric (binders "I" and "II"), the group 1 proteins are dimeric, with subunit molecular masses ranging from approximately 15 000 Da - 19 000 Da. Although present in the liver in low concentration, these binders exhibit a high specific binding of bile acids, as demonstrated by the level of incorporation of [^{125}I]-3β azidocholyl-histamine (Table 4.3). Indeed, the specific incorporation of the photoaffinity label into the group 1 Y' proteins is of the same order of magnitude as that of the glutathione S-transferases studied in this project (Table 4.3).

It is difficult to interrelate the two binders of Sugiyama et al., with any of the bile acid-binding proteins described in this thesis (Table 4.4). It is possible that binders 5C and 5D may be binders I and II; the isoelectric points of the two sets of proteins are very similar, and, although their molecular masses are different, both proteins within a pair have the same mass, i.e. binders I and II, 33 000 Da, and binders 5C and 5D, 36 200 Da. However, Sugiyama et al. (1983) reported that binders I and II had "very similar amino acid compositions", whereas the evidence presented here from peptide "maps" using both limited proteolysis in the presence of SDS and tryptic digestion with HPLC analysis (Figures 3.41, 3.45 and 3.36) shows that, although binders

5C and 5D may have some sequence homology and possess several common peptides, they are distinct proteins (see p.256). In addition, Sugiyama and his colleagues stated that binders I and II had different binding characteristics for bile acids, binder II having a greater affinity than binder I for most bile acids. Binders 5C and 5D, however, exhibit the same degree of labelling, both specific and total, (Table 4.3), approximately 20,000 counts per minute per 50 μg and 1 x 10^6 counts per minute, respectively. This would tend to suggest that both proteins have a similar affinity for [^{125}I]-3β azidocholylhistamine, and probably also for bile acids.

On balance, although it seems that binders 5C and 5D are not equivalent to binders I and II, it is difficult to understand how the latter proteins were not purified from the Y' fraction in this project, or why they were not identified by photoaffinity labelling. Alternatively, if the two pairs of binders are equivalent, as is almost certainly the case, then the data of Sugiyama *et al*. must be called into question.

PEPTIDE MAPPING OF BILE ACID-BINDING PROTEINS

Partial proteolytic digestion in the presence of SDS - Y' proteins

The limited digestion of all six Y' binders with -chymotrypsin yielded the peptide "maps" shown in Figures 3.40 and 3.41. The group 1 binders generally, and binder 5B in particular, proved somewhat resistant to digestion.

Table 4.4 Comparison of Y' binders I and II with 5C and 5D

Bile Acid-binding protein	Laboratory	Number of Subunits	Subunit Molecular Mass (Da)	Native Molecular Mass (Da)	pH from Chromatofocusing	Elution from Hydroxyapatite (K^+, mM)
[1] I	San Francisco	1	33 000	33 000	5.6	>150
II	San Francisco	1	33 000	33 000	5.5	150
[2]5C	Edinburgh	1	36 200	36 200	5.65	125
5D	Edinburgh	1	36 200	36 200	5.45	310

Note: [1]Sugiyama et al. (1983); 2C J Henderson, thesis.

However, the sequence of proteolysis in all three binders was similar, suggesting that there may be a relationship between these proteins in terms of amino acid composition and protein structure. The peptide "maps" of binders 6E and 7F were particularly alike; these two proteins are probably more closely related to each other than to binder 5B.

Partial proteolysis of binders 5B, 6E and 7F each yielded three polypeptides, of approximate molecular masses 10 000 Da, 11 000 Da and 15 000 Da, which appear to be common to all three digests.

Digestion of the group 2 binders (5C, 5D and 8C) was far more extensive when compared to the group 1 binders. Despite the fact that the proteolytic sequence was different in each case, two polypeptides were found to be common to all three proteins. The approximate molecular masses of these were 15 000 Da and 18 000 Da. However, it appears improbable that a structural relationship exists between the proteins of this group, because their degradation pathways are quite distinct.

Tryptic digestion and analysis on reverse-phase high performance liquid chromatography - Y' proteins and glutathione S-transferases

Greater resolution of peptides, and thus more information regarding the relationship(s) between the different bile acid-binding proteins, was obtained using reverse-phase HPLC, which achieves a separation of peptides based on their differential hydrophobic nature. More hydro-

phobic (and generally larger) peptides are retained for a longer time on the column and thus elute further down the solvent gradient. All six Y' binders and four glutathione S-transferases (AA, A, D and F) were peptide "mapped", both before and after photoaffinity labelling with [^{125}I]-3β azidocholylhistamine.

Non-photoaffinity labelled bile-acid binding proteins

Reference to the peptide "maps" of the group 1 and group 2 Y' binders (Figures 3.42 to 3.47) shows that binders 5B, 6E and 7F produce a smaller number of peptides following tryptic digestion. These data allow speculation on the amino acid composition of these proteins; there are probably few lysine or arginine residues (target amino acids for trypsin) and few non-polar residues such as tryptophan, phenylalanine, leucine and methionine (target amino acids for α-chymotrypsin). A comparison of the peptide "maps" of the group 1 Y' binders (Figures 3.42 to 3.44 and Table 3.9) dramatically confirms the results of the partial proteolytic digestion with α-chymotrypsin; namely, that binders 6E and 7F are more closely related to each other, having 20 peptides which co-eluted from the μ Bondapak column. In contrast, binder 5B, only appeared to share four peptides in common with binders 6E and 7F. Therefore, as a group, binders 5B, 6E and 7F have four common peptides.

However, this is on the basis of only a single separation procedure.

The relationships between the group 2 binders are not so unequivocal (Figures 3.45 to 3.47). Binder 5C shares only one or two more peptides with the other binders in the group than binder 5D does with binder 8C. Within the group, the three binders have seven common peptides, and between group 1 and group 2 there are two common peptides.

Thus, in summary, it can be said that the six Y' binders described here are genetically distinct proteins. However, it is possible that some sequence homology, and thus a limited structural relationship, may exist between these proteins.

Figures 3.48 to 3.51 show the peptide "maps" of glutathione S-transferases AA, A, D and F. The relationships between these four bile acid-binding proteins are more complex and fall outwith the scope of this thesis. However, what is of interest is the fact that in the glutathione S-transferases digests (Table 3.10), there is one peptide which appears to be common to group 1 and group 2 Y' bile acid-binders (Table 3.11), having a Rt value of approximately 24.5 min. It could thus be postulated that this common peptide might represent the site, or part of the site, at which bile acids bind to these proteins. The technique of photoaffinity labelling was used to explore this possibility further.

Photoaffinity labelled bile acid-binding proteins

The peptide "maps" of photoaffinity labelled Y' binders and glutathione S-transferases are shown in Figures

3.52 to 3.54. Peaks of radioactive labelling can clearly be seen, and the "background" radioactivity (due to non-specific photoaffinity labelling of the proteins) is low, being less than 5% of the peak totals. The fact that the peaks are sharp, with no shoulders, and occur within a single (or at most two) fractions, would tend to suggest that they are associated with single peptides, although this is obviously speculation.

There are five common peaks of labelling within the group 1 Y' binders, four of which are also common to the group 2 binders, and furthermore are common to both group 1 and group 2 binders (Table 3.12). The four peaks of labelling which are common to glutathione S-transferases AA, A, D and F (Table 3.13) are the same four which are shared by both groups of Y' binders (Table 3.14), with Rt values of approximately 27.5, 31, 33 and 34 min.

Thus, despite only a single common peptide being found between all the non-labelled bile acid-binding proteins, four common peptides were shown to exist after photoaffinity labelling of these proteins. Although the reason for this is unclear, the result itself is not surprising. It may be that there is more than one bile acid-binding site on the proteins. Perhaps more likely is that the labelled peptides represent different parts of a single site, having been labelled as the result of movement both of the label itself, and of the azido group in the label, within the binding site prior to photoactivation.

Indeed, such is the arrangement of the tertiary structures of proteins that it is unlikely that a ligand-binding site will be formed from a single peptide. If the bile acid-binding site is positioned within the proteins such that various different sections of the primary structure contribute to the site as a whole, then subsequent digestion may yield several peptides which have been photoaffinity labelled. Of the four common peptides, in all cases one, (Rt 27.5 min), is labelled to a much greater extent than the other three. This peptide may represent a major part of the bile acid-binding site.

Of the bile acid-binding proteins studied in this thesis, only binder 5B possesses an additional peak of labelling, not found in the other binders. As binder 5B exhibits the highest specific incorporation of [^{125}I]-3β azidocholylhistamine, it may be that this additional peak represents part of a high affinity bile acid-binding site, or a second bile acid-binding site, found only in binder 5B.

It is not clear why the bile acid-binding proteins yielded more peptides which appeared to be common after photoaffinity labelling. This may be due to alteration of the retention times of peptides on the reverse-phase column following the covalent attachment of the label, [^{125}I]-3β azidocholylhistamine. Alternatively, it may be that photoaffinity labelling of the proteins provides an

extra degree of sensitivity in the detection of the peptides.

Tryptic digestion and analysis on thin-layer chromatography - Y' proteins and glutathione S-transferases

Whereas the separation of the peptides by HPLC was based on their hydrophobic properties, separation by TLC was on the basis of the relative charges of the peptides. Two different solvent systems were employed, A and B (see p.215), the former giving better separation. In both these modes of separation, smaller numbers of common peptides were found. Between the group 1 and group 2 Y' binders, no common peptides were found when solvent system B was used, although this was probably due to lack of resolution using this solvent system. A single common peptide having a mobility just behind the solvent front was found between the Y' binders and the glutathione S-transferases using solvent system A. However, in solvent system A the mobility of the free, unreacted label coincided with this peak, making interpretation difficult. The data from the HPLC separation of the photoaffinity labelled peptides indicated that very little free label existed in the digests and thus any radioactive contribution from this source to the common labelled peptide described above would be minimal.

In summary, those Y' proteins shown to bind bile acids through investigation by photoaffinity labelling have been divided into two groups, based on the covalent incor-

poration of [^{125}I]-3β azidocholylhistamine into the proteins. These groups have been shown to be structurally related to each other and to a group of four glutathione S-transferases by peptide "mapping" of both labelled and non-labelled proteins.

On the basis of these results, it would appear that, although the Y' binders and glutathione S-transferases share common labelled peptides in terms of molecular size (HPLC separation), these peptides are sufficiently dissimilar in terms of amino acid composition for them to be able to be distinguished by virtue of their different molecular charges (TLC separation). The amino acid composition and sequence of such peptides should provide valuable information regarding the bile acid-binding site(s) of these proteins.

Y' PROTEINS AS CYTOSOLIC BILE ACID-BINDING PROTEINS IN RAT LIVER

The central question raised by this study is - "What role do the bile acid-binding proteins of the Y' fraction play in the cytosol of the hepatocyte"?

In comparison with the glutathione S-transferases, only the group 1 category of Y' bile acid-binding proteins approaches the same degree of specific incorporation of [^{125}I]-3β azidocholylhistamine following photoaffinity labelling (Table 4.3). Despite an apparently similar affinity for bile acids, the group 1 binders, being present in much lower concentrations in the hepatic cytosol

than the glutathione S-transferases, will contribute less to the total bile acid-binding capacity of the hepatocyte. The group 2 binders show a level of specific labelling at least an order of magnitude less than that of the glutathione S-transferases and group 1 binders, implying a lower affinity for bile acids.

These arguments are, of course, based on the assumption that the glutathione S-transferases bind bile acids *in vivo*. Doubt has recently been cast on this concept by work on the photoaffinity labelling of intact isolated hepatocytes (Abberger *et al.*, 1983). Cytosol from isolated hepatocytes photoaffinity labelled and this exhibited peaks of labelling associated with proteins identified as albumin, hydroxycholanoyl transferase and glutathione S-transferase subunits (Table 4.5). In addition, unidentified peaks were found with approximate molecular masses of 95 000 Da, 55 000 Da and 35 000 Da (Abberger *et al.*, 1983). When the intact hepatocytes were photoaffinity labelled first and the cytosol then extracted, virtually no labelling of glutathione S-transferases, hydroxycholanoyl transferase or albumin was found. However, peaks of labelling were associated with a number of proteins, including four of molecular masses similar to the Y' binders described herein - 35 000 Da, 33 000 Da, 18 000 Da and 14 000 Da.

If these proteins detected by Abberger *et al.* (1983) are Y' proteins, then it may be the case that glutathione S-

Table 4.5 Photoaffinity labelling of intact rat hepatocytes

Cytosol	Molecular masses of polypeptides revealed by photoaffinity labelling (Da)
Isolated then labelled	95 000; 67 000; 55 000; 43 000; 35 000; 27 000;
Labelled then isolated	94 000; 55 000; 35 000; 33 000; 18 000; 14 000;

Intact hepatocytes, and cytosol from intact hepatocytes, photoaffinity labelled with (7,7-azo-3α,12α-dihydroxy-5β-[3-^{3}H]cholan-24-oyl)-2-aminoethanesulphonate (Abberger et al., 1983); cytosol isolated and analysed on SDS/polyacrylamide-gel electrophoresis.

transferases do not bind bile acids to any great extent *in vivo*, and that binding proteins from the Y' fraction are responsible for the majority of bile acid-binding within the heptatocyte. Further studies by the same workers utilising photolabile derivatives of precursors of bile acids yielded the data shown in Table 4.6. These results again exhibit labelling of peaks with molecular masses similar to those bile acid-binding proteins of the Y' fraction that have been described in this thesis. Along with the results shown in Table 4.7 (Abberger *et al.*, 1983), which demonstrate a polypeptide of molecular mass 33 000 Da isolated from the mitochondria of photoaffinity labelled intact hepatocytes, these data allow speculation as to the function of Y' proteins in hepatic cytosol. It is known that the synthesis of bile acids occurs at various sites within the hepatocyte (see p.8), although it is uncertain how the reactants and products at each stage are moved within the cell between reactions. The Y' bile acid-binders, in addition to transporting conjugated and unconjugated bile acids across the hepatocyte as part of the enterohepatic circulation, may also be involved in the synthesis of bile acids, shuttling various components of the reaction chain from one part of the cell to another, for example, between Golgi-endoplasmic reticulum, cytosol and mitochondrion.

What of the glutathione S-transferases, known to bind bile acids with high affinity *in vitro*? Within the hepa-

Table 4.6 Photoaffinity labelling of intact hepatocytes with photolabile precursors of bile acids

Photoaffinity label	Molecular masses of polypeptides revealed by photoaffinity labelling(Da)
7,7-azo-5β-[12-^{3}H]cholestane-3α,12α-diol	57 000; 43 000; 14 000;
7,7-azo-3α,12α-dihydroxy-5β[3 -^{3}H]cholestan-26-oate	67 000; 60 000; 55 000; 43 000; 35 000; 14 000;
7,7-azo-3α,12α-dihydroxy-5β[3 ,12 -^{3}H]cholan-24-oate	67 000; 55 000; 43 000; 35 000; 33 000; 28 000; 18 000; 14 000;
(7,7-azo-3α,12α-dihydroxy-5β-[3 -^{3}H]-cholan-24-oyl)-2-aminoethane sulphonate	67 000; 55 000; 35 000; 33 000; 28 000; 18 000; 14 000;

Intact hepatocytes photoaffinity labelled with derivatives described above (Abberger et al., 1982). Cytosol extracted and analysed by SDS/polyacrylamide-gel electrophoresis.

Table 4.7 Photoaffinity labelling of intact hepatocytes and isolation of mitochondria

Photoaffinity label	Molecular masses of polypeptides revealed by photoaffinity labelling(Da)
7,7-azo-5β-[12-^{3}H]cholestane-3α,12α-diol	33 000; 28 000 (slight); 25 000 (slight);
7,7-azo-3α,12α-dihydroxy-5β [3 -^{3}H]cholestan-26-oate	33 000;
7,7-azo-3α,12α-dihydroxy-5β [3 ,12 -^{3}H]cholan-24-oate	33 000;
(7,7-azo-3α,12α-dihydroxy-5β-[3 -^{3}H]-cholan-24-oyl)-2-aminoethane sulphonate	33 000;

Intact hepatocytes photoaffinity labelled with derivatives described above (Abberger et al., 1982). Mitochondrial fraction isolated and analysed on SDS/ polyacrylamide-gel electrophoresis.

tocyte, the bile acid-binding site(s) of these enzymes may be occupied by other molecules, concerned with the detoxification role of the glutathione S-transferases, thus preventing them from participating in bile acid transport *in vivo*. In addition, neither the studies described here nor those of others (Abberger *et al.*, 1983) utilizing photolabile bile acid derivatives as photoaffinity probes of cytosolic bile acid-binding proteins, have shown Z protein(s) to be involved. Thus, although it may seem that there are a large number of bile acid-binding proteins present in the hepatocyte, in reality only a few of these may actually be involved in the transport of bile acids across the liver *in vivo*.

On the basis of peptide "mapping", it seems improbable that an evolutionary relationship exists between Y' proteins and the glutathione S-transferases. However, the subunit molecular masses of the group 1 Y' proteins are very similar to those of Z protein(s); a mass of 14 000 Da has been reported (Takahashi *et al.*, 1983). Further investigations, i.e. comparative peptide "mapping" of these proteins, may yield information regarding their evolutionary origins.

Alternative functions of Y' proteins

It may be that Y' proteins bind substances other than bile acids; the bile acid-binding site could consist of nothing more than a hydrophobic "pocket", to which many different compounds bind. Indeed, Y' proteins may play a

detoxification role in the hepatocyte, similar to that proposed for the glutathione S-transferases, binding such molecules as benz-pyrene and methylcholanthrene. The answer to this speculation lies in the further characterization of the bile acid-binding site(s) of these proteins, perhaps by an extension of the photoaffinity technique already applied, and also employing equilibrium dialysis and fluorescence inhibition methods.

A further clue to the role of Y′ proteins may be obtained from knowledge of their location within the hepatocyte, and their distribution in other tissues.

PHOTOAFFINITY LABELLING AND THE INVESTIGATION OF BILE ACID-BINDING PROTEINS

The use of [^{125}I]-3β azidocholylhistamine as a photoaffinity label in this study has proved successful in the search for previously undescribed bile acid-binding proteins in rat hepatic cytosol. The very high specific radioactivity of the molecule, 1900 Ci/mmol, has resulted in a more sensitive probe, as demonstrated when comparing the specific incorporations of label into proteins in this work and that of Abberger et al., (1981, 1983). The latter group demonstrated, on average, specific radioactive incorporations of approximately 500 counts per minute per 50 ug protein. This study has produced specific radioactive incorporations of between two and three orders of magnitude higher than this, 20,000 to 100,000 counts per minute per 50 ug protein.

FUTURE EXPERIMENTS

Further work on the Y' bile acid-binding proteins is necessary in order to complete their characterisation. Antisera have been raised against binders 5C and 8C. These may be of use both in the elucidation of the relation-ship(s) between the Y' binders and in "screening" for further bile acid-binders in liver and other tissues, for example, by using the "Western blot" technique. The production of antibodies against other Y' proteins, particularly the group 1 binders, awaits the purification of these proteins in sufficient quantities.

Determination of the amino acid composition of Y' proteins would be another useful step in the characterisation procedure. Similarly, the amino acid composition and sequence of the common, photoaffinity labelled, peptides of the Y' binders and glutathione S-transferases may reveal further information regarding not only the structure of the proteins but also their bile acid-binding sites.

Further study of the Y' binders by photoaffinity labelling, employing a range of photolabile bile acid derivatives, may yield more data concerning the bile acid-binding capabilities of these proteins, and of their relationship to one another.

APPENDIX I

A) CHEMICALS AND SUPPLIERS

This Appendix contains a full list of chemicals, radiochemicals and solvents used in this study, and arranged according to supplier.

Amersham International, Amersham, Bucks, U.K.

(Carboxy-^{14}C)-lithocholic acid (approximately 59 Ci/mmol); [^{125}I]-sodium iodide (approximately, 1900 Ci/mmol); tauro(carboxyl-^{14}C)-cholic acid, sodium salt (approximately 61 Ci/mmol).

Bio-Rad Laboratories Ltd., Watford, Herts, U.K.

Biogel HT

Macfarlane Robson Ltd., Glasgow, U.K. (BDH Agents)

Acetone; acetic acid; acrylamide; 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris); ammonium persulphate; anisaldehyde; butan-2-ol; chloramine T; 1-chloro-2,4-dinitrobenzene; chloroform; dicyclohexylcarbodimide; diethyl ether; dimethyl formamide; dimethylsuphoxide; dipotassium hydrogen orthophosphate, disodium hydrogen orthophosphate; ethanol; ethyl acetate; histamine; hydrochloric acid; N-hydroxy succinimide; isopropyl-alcohol; 2-mercaptoethanol; methanol; NN -methylene bisacrylamide; potassium dihydrogen orthophosphate; pyridine; sodium azide; sodium chloride; sodium dihydrogen orthophosphate; sodium dodecyl sulphate; sodium metabisulphite; sodium sulphate; sucrose; sulphuric acid;

tetrahydrofuran; NNN'N'-tetramethyl ethylenediamine; toluene; trichloroacetic acid; triton X-100; 2,2,4-trimethylpentane.

Maybridge Chemical Co., Tintagel, Cornwall, U.K.

Cholic acid.

Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K.

p-bis(2-(5-phenyloxazolyl))-benzene.

Packard Instrument Co., Inc., Illinois, U.S.A.

2,5-diphenyloxazole.

Rathburn Chemicals Ltd., Walkerburn, Peeblesshire, Scotland

Acetonitrile; methanol; water (HPLC grade); trifluoroacetic acid (sequencer grade).

Sigma Chemical Co.(London) Ltd., Poole, Dorset, U.K

Blue dextran; bovine serum albumin; α-chymotrypsin; α-chymotrypsinogen A; Coomassie brilliant blue R; DEAE-Sephadex A-50; glutathione (reduced); lithocholic acid; myoglobin; ovalbumin; polybuffer 74; polybuffer exchanger 94; Sephadex G-25 fine, Sephadex G-75 superfine; Sephadex G-100 fine; SDS/molecular mass calibration kit; trypsin.

Scientific Instrument Centre Ltd., London, U.K.

Visking dialysis tubing (molecular cut off approximately 10 000 Da).

B) FURTHER PURIFICATION OF CHEMICALS

Listed here are those chemicals which it was necessary to further purify. Also given are the procedures used for purification.

Cholic acid - recrystallised four times from methanol.

Dimethylsulphoxide - dried by refluxing with sodium hydroxide pellets for 30 min and collected by distillation at 189°C.

Pyridine - dried by refluxing with potassium hydroxide pellets for 2 h and collected by distillation at 114°C.

Sodium tetrahydrofuran - dried over sodium wire.

p-toluene sulphonyl chloride - recrystallised hot from petroleum ether (40-60°C) as white monoclinic crystals.

APPENDIX II

This appendix contains a list of the techniques used in the characterisation of the intermediates in the synthesis of [^{125}I]-3βazidocholylhistamine.

Ultraviolet

Ultraviolet spectra of bile acid-derivatives were measured on a Pye-Unicam SP 1800 double-beam spectrophotometer (Pye-Unicam Ltd., Cambridge, U.K.).

Infra-red

Infra-red spectra of bile acid derivatives were measured on a Pye-Unicam SP 200 double-beam spectrophotometer (Address as above).

Melting points

Melting points were determined with a Reichert Thermopan Microscope (C. Reichert, Optische Werke AGm, Wien, Austria).

Nuclear magnetic resonance

Proton and ^{13}C nuclear magnetic resonance spectra were measured on a Bruker 200 MH_3 instrument (Bruker, Zurich, Switzerland). The solvent for the proton scans was deuterated chloroform, ($CDCl_3$) and for ^{13}C, dimethyl sulphoxide-d_6.

APPENDIX III

This appendix lists the procedure used in the preparation of rat hepatic cytosol for the purification of Y' proteins.

Preparation of cytosol

Male Wistar rats were killed under ether anaesthetic and the livers removed immediately into ice-cold buffer H. After weighing, the livers were chopped finely and homogenized in buffer H (volume = twice total liver weight in ml). The homogenate was centrifuged for 30 min at 18,000 g, 4°C. After decantation, the supernatant was recentrifuged for 60 min at 100,000 g, 4°C. The supernatant was then filtered through a glass wool plug and stored on ice until the purification procedure was begun.

APPENDIX IV

This appendix lists all the abbreviations used in the thesis. The abbreviations are defined both here and the first time they appear in the text.

ANS	1-anilino-8-naphthalene sulphonate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CPM	counts per minute
DEAE	diethylaminoethyl
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HMG CoA	3-hydroxy-3-methyl-glutaryl coenzyme A
pI	isoelectric point
POPOP	1,4-di(2-(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
Rt	retention time
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TLC	thin layer chromatography
Tris	[2-amino-2-(hydroxymethyl)propane-1,3-diol]

REFERENCES

Abberger, H., Buscher, H-P., Gerok, W., Kramer, W. and Kurz G. (1978) Hoppe-Seylers Z. Physiol. Chem. 359, 251.

Abberger, H., Bickel, U., Buscher, H-P., Fuchte, K. and Gerok, W. (1981) In: Bile Acids and Lipids (Eds. Paumgartner, G., Stiehl, A. and Gerok, W.) pp. 233-246, MTP Press, Lancaster, England.

Abberger, H., Buscher, H-P., Fuchte, K., Gerok, W., Giese, U., Kramer, W., Kurz, G. and Zanger, U. (1983) In: Bile acids and cholesterol in Health and Disease (Eds. Paumgartner, G., Stiehl, A. and Gerok, W.) pp. 77-87, MTP Press, Lancaster, England.

Accatino, L. and Simon, R.F. (1976) J. Clin. Invest. 57, 496-528.

Admirand, W.H. and Small, D.M. (1968) J. Clin. Invest. 47, 1043-1052.

Albright, E.C., Larson, F.C. and Deiss, W.P. (1953) Proc. Soc. Exp. Biol. Med. 84, 240-244.

Alme, B., Bremmelgaard, A., Sjovall, J. and Thomassen, P. (1977) J. Lipid Res. 18, 339-362.

Anderson, K.E., Kok, E. and Javitt, N.B. (1972) J. Clin. Inv. 51, 112-117.

Anwer, M.S., Kroker, R., Hegner, D. and Petter, A. (1977) Hoppe-Seylers Z. Physiol. Chem. 358, 543-553.

Anwer, M.S. and Hegner, D. (1978a) Hoppe-Seylers Z. Physiol. Chem. 359, 181-192.

Anwer, M.S. and Hegner, D. (1978b) Hoppe-Seylers Z. Physiol. Chem. 359, 1027-1030.

Aries, V. and Hill, M.J. (1970) Biochim. Biophys. Acta, 202, 526-534.

Back, P., Hamprecht, B. and Lynen, F. (1969) Arch. Biochem. Biophys. 133, 11-21.

Back, P., Spaczyinski, K. and Gerok, W. (1974) Hoppe-Seylers Z. Physiol. Chem. 355, 749-752.

Back, P. (1975) In: Advances in bile acid research (Eds. Matern, S., Hachenschmidt, J., Back, P. and Gerok, W.) pp. 149-152, Stuttgart, New York, Schattauer.

Back, P. (1976) Hoppe-Seylers Z. Physiol. Chem. 357, 213-217.

Baker, A.L., Wood, R.A.B., Moosa, A.R., and Boyer, J.L. (1979) J. Clin. Invest. 64, 312-320.

Balabaud, G., Kron, K.A. and Gumucro, J.J. (1977) J. Lab. Clin. Med. 89, 393-399.

Barnett, J. and Reichstein, T. (1938) Helv. Chim. Acta 21, 926-938.

Barnwell, S.G., Lowe, P.J. and Coleman, R. (1984) Biochem. J. 220, 723-731.

Barrow, G.M. (1973) In: Physical Chemistry Int. Stud. Ed., Chap. 13, McGraw-Hill, Kagakushwa Ltd., Tokyo, Japan.

Bass, N.M., Kirsch, R.E., Tuff, S.A., Marks, I. and Saunders, S.J. (1977) Biochim. Biophys. Acta 588, 70-80.

Baum, B.J., Johnson, L.S., Franzblau, C. and Troxler, R.F. (1975) J. Biol. Chem. 250, 1464-1471.

Bayley, H. and Knowles, J.R. (1977) Methods Enzymol. 46, 69-114.

Beckett, G.J., Hunter, W.M. and Percy-Robb, I.W. (1978) Clin. Chim. Acta 88, 257-265.

Beckett G.J., Corrie, J.E.T. and Percy-Robb, I.W. (1979) Clin. Chim. Acta 93, 145-150.

Beppo, M., Terao, T. and Osawa, T. (1975) J. Biochem. (Tokyo) 78, 1013.

von Bergman, K., Schultheiss, H.R., Paumgartner, G. and Preisig, R. (1975) Schweiz Med. Wschr. 105, 413-415.

Bergstrom, S. (1962) Fed. Proc. 21(4), Pt2,28.

Bisson, R. Azzi, A., Gutweniger, H., Colonna, R. Mentecucco, C. and Zanotti, A. (1978) J. Biol. Chem. 253, 1874-80.

Bjorkhem, I. and Danielsson, H. (1967) Eur. J. Biochem. 2, 403-413.

Bjorkhem, I. and Danielsson, H. (1974) Mol. Cell. Biochem. 4, 79-95.

Blitzer, B.L. and Boyer, J.L. (1982) Gastroenterology 82, 346-357.

Borgstrom, B., Dahlquist, A., Lundh, G., and Sjovall, J. (1957) J. Clin. Invest. 36, 1521-1536.

Botham, K.M., Beckett, G.J., Percy-Robb, I.W. and Boyd, G.S. (1979) Eur. J. Biochem. 103, 299-305.

Botham, K.M., Lawson, M.E., Beckett, G.J., Percy-Robb, I.W. and Boyd, G.S. (1980) Biochim. Biophys. Acta 665,81-87.

Botham, K.M., Lawson, M.E., Beckett, G.J., Percy-Robb, I.W. and Boyd, G.S. (1981) Biochim. Biophys. Acta 666, 238-245.

Boyd, G.S., Scholan, N.A. and Mitton, J.R. (1969) Adv. Exp. Med. Biol. 4, 443-456.

Boyd, G.S. and Percy-Robb, I.W. (1971) Am. J. Med. 51, 580-587.

Boyer, J.L. and Klatskin, G. (1970) Gastroenterology 59, 853-859.

Boyer, J.L. and Bloomer, R. (1974) J. Clin. Invest. 54, 773-781.

Boyer, J.L., Itabishi, M. and Hruban, Z. (1979) In: Liver:Quantitative aspects of structure and function (Eds. Preisig, R. and Bircher, J.) pp.163-178 Editio Cantor, Aulendorf, F.R.G.

Boyer, J.L. (1980) Physiol. Rev. 60, 303-326.

Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

Bremer, J. (1956) Acta Chem. Scand. 10, 56-71.

Browne, D.J., Hixson, S.S. and Westheimer, F.H. (1971) J. Biol. Chem. 246, 4477-4484.

Bundy, R. Mauskopf, J., Walker, J.T. and Lack, L. (1977) J. Lipid Res. 18, 389-395.

Burckhardt, G., Kramer, W., Kurz, G. and Wilson, F.A. (1983) J. Biol. Chem. 258, 3618-3622.

Cantrell, G.W. and Yielding, K.L. (1977) Photochem. Photobiol. 25, 189-191.

Carey, C.M. and Small, D.M. (1967) Ann. Rev. Med. 18, 333-376.

Chen, L.J., Bolt, R.J. and Admirand, W.H. (1977) Biochim. Biophys. Acta 480, 219-227.

Cheng, S. and Levy, D. (1979) J. Biol. Chem. 255, 2637-2640.

Chowdhry, V., Vaughan, R. and Westheimer, F.H. (1976) Proc. Nat. Acad. Sci (USA) 73, 1406-1408.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102-1106.

Cooperman, B.S. and Brunswick, D.J. (1973) Biochemistry 12, 4079-4084.

Cooperman, B.S. (1976) In: Aging, carcinogenesis and radiation biology (Ed. Smith, K.C.) pp. 315-340, Plenum Press, New York.

Cowen, A.E., Korman, M.G., Hofmann, A.F., and Cass, O.W. (1975a) Gastroenterology 69, 59-66.

Cowen, A.E., Korman, M.G., Hofmann, A.F., Cass, O.W. and Coffin, S.B. (1975b) Gastroenterology 69, 67-76.

Creed,D. (1974) Photochem. Photobiol, 19, 459-462.

Cronholm, T. and Sjovall, J. (1967) Eur. J. Biochem. 2, 375-383.

Dam, M. and Hegardt, F.G. (1971) Z. Ernachrungswiss, 10, 239-246.

Danielsson, H. and Einarsson, K. (1969) Biol. Basis Med. 5, 279-315.

Danielsson, H. (1972) Steroids 20, 63-72.

Danielsson, H. (1973) In: The bile acids: Chemistry, Physiology and Metabolism (Eds. Nair, P.P. and Kritchevsky, D.) Vol. III, pp. 1-32 N.Y. Plenum Press.

Danielsson, H. and Johansson, G. (1974) Gastroenterology 67, 126-134.

Danielsson, H. and Sjovall, J. (1975) Ann. Rev. Biochem. 44, 233-253.

Davenport, H.W. (1966) In: Physiology of the digestive tract (2nd Ed.) 245p., Yearbook Medical Publishers, Chicago.

Davis, R.A., Highsmith, W.E., Malone-McNeal, M., Archambault-Schexnayder, J.A. and Kuan, J-C.W. (1983a) J. Biol. Chem. 258, 4079-4082.

Davis, R.A., Hyde, P.M., Kuan, J-C.W., Malone-McNeal, M. and Archambault-Schexnayder, J.A. (1983b) J. Biol. Chem. 258, 3661-3667.

Dempsey, M.E., McCoy, K.E., Baker, H.N., Dmitriado-Vafiadou, A., Lorsbach, T. and Howard, J.B. (1981) J. Biol. Chem. 256, 1867-1873.

Dietschy, J.M. (1966) Gastroenterology 50, 692-707.

Dietschy, J.M., Salomon, H.S. and Siperstein, M.D. (1966) J. Clin. Invest. 65, 832-846.

Dickenson, A.B., Gustafsson, B.E. and Norman, A. (1971) Acta Path. Microbiol. Scand. 79, 691-698.

von Dippe, P., Drain, P. and Levy D. (1983) J. Biol. Chem. 258, 8890-8895.

von Dippe, P. and Levy, D. (1983) J. Biol. Chem. 258, 8896-8901.

Dowling, R.H. (1972) Gastroenterology 62, 122-140.

Drasar, B.S., Hill, M.J. and Shiner, M. (1966) Lancet, 1, 1237-1238.

Drasar, B.S. and Shiner, M. (1969) Gut, 10, 812-819.

Einarrson, K., Hellstrom, K. and Kellner, M. (1973) Metabolism 22, 1477-1483.

Einarsson, K. and Hellstrom, K. (1974) Clin. Sci. Mol. Med. 46, 183-190.

Elliot, W.H. (1955) Biochim. Biophys. Acta 17, 440-441.

Elliot, W.H. and Hyde, P.M. (1971) Am. J. Med. 51, 568-579.

Eneroth, P. (1963) J. Lipid Res. 4, 11-16.

Eneroth, P., Gordon, B, Ryhage, R. and Sjovall, J. (1966a) J. Lipid Res. 7, 511-523.

Eneroth, P., Gordon, B. and Sjovall, J. (1966b) J. Lipid Res. 7, 524-530.

Erlinger, S., Dhumeaux, D., Berthelot, P. and Dumont, M. (1970) Am. J. Physiol. 219, 416-422.

Escher, E. and Schwyser, P. (1975) Helv. Chim. Acta 58, 1465-1471.

Fleet, G.W.J., Porter, R.R. and Knowles, J.R. (1969) Nature 224, 511-512.

Fricker, G., Kramer, W., Buscher, H.P., Gerok, W. and Kurz, G. (1982) Hoppe-Seylers Z. Physiol. Chem. 363, 897.

Frohling, W. and Stiehl, A. (1976) Eur. J. Clin. Invest. 6, 67-74.

Frohling, W., Stiehl, A., Czygan, P. and Kommerell, B. (1977) Biochem. Biophys. Acta 444, 525-530.

Gallagher, K., Mauskopf, J., Walker, J.T. and Lack, L. (1976) J. Lipid Res. 17, 572-577.

Garbutt, J.T. and Kenney, J.T. (1972) J. Clin. Inv. 51, 2781-2789.

Glasinovic, J.C., Dumont, M., Duval, M. and Erlinger, S. (1975) J. Clin. Invest. 55, 419-426.

Glasser, J.E., Weiner, I.M. and Lack, L. (1965) Am. J. Physiol. 208, 359-362.

Glazer, A.N., Delange, R.J. and Sigman, D.S. (1976) In: Laboratory Techniques in Biochemistry and Molecular Biology (Eds. Work, T.S. and Work, E.) pp. 1-205, North-Holland Publishing Company, Amsterdam.

Goldsmith, M.A., Huling, S. and Jones, A.L. (1983) Gastroenterology 84, 978-986.

Gorbach, S.L., Plant, A.G., Nahas, L., Weinstein, L., Spanknebel, G. and Levitan, R. (1967) Gastro. 53, 856-867.

Gorbach, S.L. and Tabaqchali, S. (1969) Gut 10, 963-972.

Grandjean, E.M., Karlaganis, G. Noellp, U., Hwegli, H, Roesler, H. and Paumgartner, G. (1978) in The Liver - 3rd International Gstaad Symposium (Eds. Preisig, R. and Paumgartner, G.) pp. 255-262, Editio Cantor, Aulendorf, F.R.G.

Grundy, S.M., Ahrens, E.H.Jr. and Miettinen, T.A. (1965) J. Lipid Res. 6, 397-410.

Grundy, S.M., Metzger, A.L. and Adler, R.D. (1972) J. Clin. Invest. 51, 3026-3043.

Guillory, R.J., Rayner, M.D. and D'Arrigo, J.S. (1977) Science 196, 883-885.

Gustaffson, B.E. and Norman, A. (1962) Proc. Soc. Exp. Biol. 110, 387-389.

Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M. and Jakoby, W.B. (1974) Proc. Nat. Acad. Sci. (USA) 71. 3879-3882.

Hanson, R.F. (1971) J. Clin. Invest. 50, 2015-2025.

Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1979) Biochem. J. 181, 699-708.

Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1980) Biochem. J. 185, 83-87

Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1981) Biochem. J. 197, 491-502.

Hayes, J.D. and Chalmers, J. (1983) Biochem. J. 215, 581-588.

Hayes, J.D. (1984) Biochem. J. (In press).

Hepner, G.W., Hofmann, A.F. and Thomas, P.J. (1972a) J. Clin. Invest. 51, 1889-1897.

Hepner, G.W., Hofmann, A.F. and Thomas, P.J. (1972b) J. Clin. Invest. 51, 1898-1905.

Hexter, C.S. and Westheimer, F.H. (1971) J. Biol. Chem. 246, 3928.

Hislop, I.G., Hofmann, A.F. and Schoenfield, L.J. (1967) J. Clin. Invest. 46, 1070-1071.

Hixson, S.S. and Hixson, S.H. (1972) J. Org. Chem. 37, 1279.

Hofmann, A.F. and Borgstrom, B. (1962) Fed. Proc. Amer. Soc. Exp. Biol. 21, 43-50.

Hofmann, A.F. (1963) Biochem. J. 89, 57-68.

Hofmann, A.F. and Small, D.M. (1967) Ann. Rev. Med. 18, 333-376.

Hofmann, A.F. and Poley, J.R. (1972) Gastroenterology 62, 918.

Hofmann, A.F. (1976) In: Lipid absorption: Biochemical and Clinical Aspects. (Eds. Rommel, K., Goebbel, H. and Behmer, R.) 352 pp. University Park Press, Baltimore.

Holt, P.R. (1964) Am. J. Physiol. 207, 1-7.

Holt, P.R. (1972) Arch. Intern. Med. 130, 574-583.

Hummel, J.P. and Dreyer, W.J. (1962) Biochim. Biophys. Acta 63, 530-532.

Jakoby, W.B., Habig, W.H., Keen, J.H., Ketley, J.N. and Pabst, M.J. (1976) In: Glutathione: Metabolism and function (Eds. Arias, I.M. and Jakoby, W.B.) pp. 189-202, Raven Press, New York.

Jakoby, W.B. and Wilchek, M. (1977) Methods Enzymol. Vol.46, 774 pp. Academic Press, Inc., New York.

Javitt, N.B. and Emerman, S. (1968) J. Clin. Invest. 47, 1002-1008.

Javitt, N.B. (1975) In: Diseases of the liver (Eds. Schiff, L.) p. 111, J.B.Lippincott Co., Philadelphia and Toronto.

Jones, A.L., Schmucker, D.L., Adler, R.D. Ockner, R.K. and Mooney, J.S. (1979) In: Liver: Quantitative aspects of structure and function (Eds. Preisig, R., Bircher, J. and Paumgartner, G.) pp. 36-51, Editio Cantor, Aulendorf, F.R.G.

Jones, A.L., Schmucker, D.L., Mooney, J.S. and Ockner, R.K. (1976b) Gastroenterology 71, 1050-60.

Jones, A.L. and Spring-Mills, E. (1977) In: Histology (Eds. Weiss, L. and Greep, R.O.) pp. 701-747 McGraw-Hill Book Co., New York.

Jones, A.L., Schmucker, D.L., Mooney, J.S., Ockner, R.K. and Adler, R.D. (1979). Lab. Invest. 40, 512-517.

Jones, A.L., Schmucker, D.L. and Renston, P.H. (1980a) Dig. Dis. Sci. 25, 609-629.

Jones, A.L., Hradek, G.T., Renston, R.H., Wong, K.Y., Karlaganis, G. and Paumgartner, G. (1980b) Am. J. Physiol. 238, G233-237.

Jones, M. Jr. and Moss, R.A. (Eds.) "Carbene" Wiley (Interscience) New York, Vol.I (1973); Vol. II (1975).

Jori, G. (1973) Anals de Academios Brasileira de Ciencias 45, 33-44.

Jori, G. and Spikes, J.D (1977) In: Science of Photobiology, (Ed. Smith, K.C.) p.87, Plenum Press, New York.

Kakis, G., Yousef, I.M. and Fisher, M.M. (1957) Gastroenterology 72, 1178.

Kaplowitz, N., Percy-Robb, I.W. and Javitt, N.B. (1973) J. Exp. Med. 138, 483-487.

Katzenellenbogen, J.A., Johnson, H.J. Jr., Carlson, K.E. and Myers, H.N. (1974) Biochemistry 13, 2986-2994.

Kelly, R.L. and Doisy, E.A. (1964) Fed. Proc. 23, 173.

Ketterer, B., Tipping, E., Hackney, J.F. and Beale, D. (1976) Biochem. J. 155, 511-521.

Kiefer, H., Lindstrom, J., Lennox, E.S. and Singer, S.J. (1970) Proc. Nat. Acad. Sci.(USA) 67, 1688-1694.

Killenberg, P.G. and Jordan, J.T. (1978) J. Biol. Chem. 253, 1005-1010.

Killenberg, P.G. (1978) J. Lipid Res. 19, 24-31.

Knowles, J.R. (1972) Accounts Chem. Res. 5, 155-160.

Krag, E. and Phillips, S.F. (1974) J. Clin. Invest. 53, 1686-1694.

Kramer, W, Buscher, H-P., Gerok, W. and Kurz, G. (1979) Eur. J. Biochem. 102, 1-9.

Kramer, W. Bickel, U., Buscher, H-P, Gerok, W. and Kurz, G. (1980) Hoppe-Seylers Z. Physiol. Chem. 361, 1307.

Kramer, W., Bickel, U., Buscher, H-P, Gerok, W. and Kurz, G. (1982) Eur. J. Biochem. 129, 13-24.

Kramer, W., Burckhardt, G., Wilson, F.A. and Kurz, G. (1983) J. Biol. Chem. 258, 3623-3627.

Kramer, W. and Kurz, G. (1983) J. Lipid Res. 24, 910-923.

Lack, L. and Weiner, I.M. (1961) Am. J. Physiol. 200, 313-317.

Laemmli, U.K. (1970) Nature (London) 227, 680-685.

Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1969) J. Clin. Invest. 48, 2156-2167.

Lewis, R. and Gorbach, S. (1972) Arch. Intern. Med. 130, 545-549.

Loof, L. and Wengle, B. (1978) Biochim. Biophys. Acta 530, 451-460.

Maasen, J.A. and Muller, W. (1974) Proc. Nat. Acad. Sci.(USA) 71, 1277-1280.

Maizel, J.V. (1971) In: Methods in Virology, Vol. 5 (Eds. Maramorosch, K. and Koprowski, H.) p.179-246, Academic Press, New York and London.

Mannervik, B. (1984) Isoenzymes of glutathione S-transferase. Advances in Enzymology. (In press).

Matern, S. and Gerok, W. (1979) Rev. Physiol. Biochem. Pharmacol. 85, 125-204.

Mendelsohn, D., Mendelsohn, L. and Stable, E. (1966) Biochemistry 5, 1286-1290.

Meyer, J.H. and Grossman, M.I. (1972) In: Gastro-intestinal Hormones, (Ed. Damborg, L.) pp. 43-55 Stuttgart:Thieme.

Mitropoulis, K.A. and Myant, N.B. (1967) Biochem. J. 103, 472-479.

Mosbach, E.H. and Salem, G. (1974) Am. J. Dig. Dis. 19, 920-929.

Myant, N.B. and Mitropoulis, K.A. (1977) J. Lipid Res. 18, 135-153.

Neiderhiser, D.H. and Roth, H.P. (1968) Proc. Soc. Exp. Biol. Med. 128, 221-225.

Nielsen, P.E., Hansen, J.B., Thomson, T. and Buchardt, O. (1983) Experientia 39, 1063-1072.

Norman, A. (1970) Scand. J. Gastroenterology 5, 231-236.

Ockner, R.K., Manning, J.A., Poppenhausen, R.B. and Ho, W.K.L. (1972) Science (Washington, D.C.) 177, 56-58.

Ockner, R.K. and Isselbacher, K.J. (1974) In: Rev. Physiol. Biochem. Pharmacol. 71, pp. 107-146, Berlin, Heidelberg, New York:Springer.

Ohkuma, S. and Kuriyama, K. (1982) Steroids 39, 7-21.

O'Maille, E.R.L., Richards, T.G. and Short, A.H. (1965) J. Physiol. 180, 67-69.

O'Maille, E.R.L., Richards, T.G. and Short, A.H. (1967) J. Physiol. 189, 337-350.

O'Moore, R.R.L. and Percy-Robb, I.W. (1973) Clin. Chim. Acta 43, 39-47.

Palmer, R.H. (1964) Nature (London) 201, 1134-1135.

Palmer, R.H. (1967) Proc. Nat. Acad. Sci.(USA) 58, 1047-1050.

Pattinson, N.R., Collins, D. and Campbell, B. (1980) J. Chrom. 187, 409-412.

Pattinson, N.R. (1981a) Anal. Biochem. 115, 424-427.

Pattinson, N.R. (1981b) Biochim. Biophys. Acta 667, 70-76.

Pattinson, N.R. (1981c) Biochem. Biophys. Res. Comm. 102, 403-410.

Paumgartner, G., Herz, R., Sauter, K. and Schwarz, H.P. (1974) Naunyn-Schmiedebergs Arch. Pharmacol. 285, 165-174.

Paumgartner, G. and Reichen, J. (1975) Experientia 31, 306-308.

Paumgartner, G., Reichen, J., von Bergmann, K. and Preisig, R. (1975) Bull. N.Y. Acad. Med. 51, 455-471.

Percy-Robb, I.W. (1975) Essays Medical Biochem. 1, (Eds Marks, V. and Hales, C.N.) p.59-80. The Biochemical Society and Association of Clinical Biochemists, London.

Pickett, C.B., Telakowski-Hopkins, C.A., Ding, G. J-F., Argenbright, L. and Lu, A.Y.H. (1984) J. Biol. Chem. 258, 5182-5188.

Polokoff, M.A. and Bell, R.M. (1977) J. Biol. Chem. 252, 1167-1171.

Pope, J.L., Parkinson, T.M. and Olson, J.A. (1966) Biochim. Biophys. Acta 130, 218-232.

Poupon, R.E., Poupon, R.Y., Dumont, M. and Erlinger, S. (1976) Eur. J. Clin. Invest. 6, 431-437.

Prager, G.N., Voigt, W. and Hsia, S.L. (1973) J. Biol. Chem. 248, 8442-8448.

Reichen, J. and Paumgartner, G. (1975) Gastroenterology 68, 132-136.

Reichen, J. and Paumgartner, G. (1976) Am. J. Physiol. 231, 734-742.

Reichen, J. Preisig, R. and Paumgartner, G. (1977) In: Bile Acid Metabolism in Health and Disease (Eds. Paumgartner, G. and Stiehl, A.) pp. 113-123, MTP Press, Lancaster, England.

Renston, R.H., Jones, A.L., Christiansen, W.D. and Hradek, G.T. (1980a) Science (Washington, D.C.) 208, 1276-1278.

Renston, R.H., Maloney, D.G., Jones, A.L., Hradek, G.T., Wong, K.T. and Goldfine, I.D. (1980b) Gastroenterology 78, 1373-1378.

Rosenblit, P.D. and Levy, D. (1977) Biochem. Biophys. Res. Comm. 77, 95-103.

Ruoho, A.E., Kiefer, H., Roeder, P. and Singer, S.J. (1973) Proc. Nat. Acad. Sci.(USA) 70, 2567-2571.

Rudman, D. and Kendall, F.E. (1957) J. Clin. Invest. 36, 538-542.

Rudnick, G., Kaback, H.R. and Weil, R. (1975) J. Biol. Chem. 250, 1371-1375.

Schiff, E.R., Small, N.C. and Dietschy, J.M. (1972) J. Clin. Invest. 51, 1351-1362.

Scharschmidt, B.F. (1982) In: Hepatology, A Textbook of Liver Diseases (Eds. Zakim, D. and Boyer, T.D.) pp.297-351, W.B. Saunders Company, Philadelphia and London.

Schoenfield, L.J., Bonorris, G.G. and Granz, P. (1973) J. Lab. Clin. Med. 82, 858-868.

Schwarz, L.R., Burr, R., Schwenk, M. Pfaff, E. and Greim, H. (1976) Eur. J. Biochem. 71, 369-377.

Scully, N.C. and Mantle, T.J. (1981) Biochem. J. 193, 367-370.

Shafer, J. Baronowksy, P., Laursen, R., Finn, F. and Westheimer, F.H. (1966) J. Biol. Chem. 241, 421.

Shefer, S. Hauser, S. and Mosbach, E.H. (1968) J. Lipid Res. 9, 328-333.

Shefer, S., Hauser, S, Bekersky, I. and Mosbach, E.H. (1969) J. Lipid Res. 10, 646-655.

Shefer, S., Hauser, S., Bekersky, K. and Mosbach, E.H. (1970) J. Lipid Res. 11, 404-411.

Shefer, S., Hauser, S, Lapar, V. and Mosbach, E.H. (1972) J. Lipid Res. 13, 402-412.

Shefer, S., Hauser, S, Lapar, V. and Mosbach, E.H. (1973a) J. Lipid Res. 14, 573-580.

Shefer, S., Hauser, S., Lapar, V. and Mosbach, E.H. (1973b) J. Lipid Res. 14, 400-405.

Shefer, S., Nicolau, G. and Mosbach, E.H. (1975) J. Lipid Res. 16, 92-96.

Simmonds, W.J., Korman, M.G., Go, V.L.W. and Hofmann, A.F. (1973) Gastroenterology 65, 705-711.

Simmonds, W.J. (1974) In: Gastrointestinal Physiology, MTP International Review of Science, Physiology Series I, Baltimore University Park Press.

Simons, P.C. and Vander Jagt, D.L. (1977) Anal. Biochem. 82, 334-341.

Singh, A., Thornton, E.R. and Westheimer, F.H. (1962) J. Biol. Chem. 237, PC 3006-8.

Sjovall, J. (1960) Clin. Chim. Acta 5, 33-41.

Small, D.M. (1970) Adv. Internal Med. 16, 243-264.

Small, D.M. (1971) In: The Bile Acids, Chemistry, Physiology and Metabolism (Eds. Nair, P.P. and Kritchevsky, D.) Vol. 1, pp. 249-356, New York, London, Plenum Press.

Small, D.M., Dowling, R.H. and Redinger, R.H. (1974) Arch. Internal Med. 130, 552-573.

Smallwood, R.A., Iser, J.H. and Hofmann, N.E. (1974) In: Advances in Bile Acid Research (Eds. Matern, S., Hackenschmidt, J., Back, P. and Gerok, W.) pp. 229-232, Schattauer-Verlag GmbH, Stuttgart.

Smith, R.A.G. and Knowles, J.R. (1975a) J. Am. Chem. Soc. 95, 5072-5073.

Smith, R.A.G. and Knowles, J.R. (1975b) J. Chem. Soc., Perkins Trans. 2, 686-694.

Spenny, G.J., Tobin, M.M., Mihas, A.A., Gibson, R.G., Hirschowitz, B.I., Johnson, B.J. and Tauxe, W.M. (1978) Gastroenterology 76, 272-278.

Sperber, I. (1963) In: Proc. 1st Int. Pharmacol. Meeting, Oxford, Pergamon 4, 137-143.

Sperber, I. (1965) In: The Biliary System (Ed. Taylor, W.) pp.457-467, Blackwell, Oxford.

Strange, R.C., Nimmo, I.A. and Percy-Robb, I.W. (1977a) Biochem. J. 162, 659-664.

Strange, R.C., Cramb, R., Hayes, J.D. and Percy-Robb, I.W. (1977b) Biochem. J. 165, 425-429.

Strange, R.C., Beckett, G.J. and Percy-Robb, I.W. (1979a) Biochem. J. 178, 71-78.

Strange, R.C., Chapman, B.J., Johnston, J.D., Nimmo, I.A. and Percy-Robb, I.W. (1979b) Biochim. Biophys. Acta 573, 535-545.

Strange, R.C., Nimmo, I.A. and Percy-Robb, I.W. (1979c) Biochim. Biophys. Acta 588, 70-80.

Strange, R.C. (1981) Biochem. Soc. Trans. 9, 170-174.

Sugiyama, Y., Yamada, T. and Kaplowitz, N. (1982) Biochim. Biophys. Acta 709, 342-352.

Sugiyama, Y., Yamada, T. and Kaplowitz, N. (1983) J. Biol. Chem. 258, 3602-3607.

Sugiyama, Y., Tadataka, Y. and Kaplowitz, N. (1982) Biochem. J. 203, 377-381.

Tabaqchali, S. (1970) Scand. J. Gastroenterology 5 (Suppl. 6) 139-163.

Takahashi, K., Odani, S. and Ono, T. (1983) Eur. J. Biochem. 136, 589-601.

Taniguchi, S., Hoshita, N. and Okuda, K. (1973) Eur. J. Biochem. 40, 607-617.

Teem, M.V. and Phillips, S.F. (1972) Gastroenterology 62, 261-267.

Tyor, M.P., Garbatt, J.T. and Look, L. (1971) Am. J. Med. 51, 614-626.

Vander Jagt, D.L., Wilson, S.P. and Heidrich, J.E. (1981) FEBS Letters 136, 319-321.

Vander Jagt, D.L., Wilson, S.P., Dean, V.L. and Simons, P.C. (1982) J. Biol. Chem. 257, 1997-2001.

Vaughn, R.J. and Westheimer, F.H. (1969) J. Am. Chem. Soc. 91, 217-218.

Vessey, D.A. and Zakim, D. (1977) Biochem. J. 163, 357-362.

Vessey, D.A. and Grissey, M.H. and Zakim, D. (1977) Biochem. J. 163, 181-183.

Vessey, D.A. and Zakim, D. (1981) Biochem. J. 197, 321-325.

Wheeler, H.O. (1972) Arch. Int. Med. 130, 533-541.

Wilson, F.A. and Dietschy, J.M. (1972) J. Clin. Invest. 51, 3015-3025.

Wilson, F.A. and Treanor, L.L. (1975) Biochim. Biophys. Acta 406, 280-293.

Wolkoff, A.W., Goresky, C.A., Selhn, J., Gatmaitan, Z. and Arias, I.M. (1979) Am. J. Physiol. 236, E638-648.

Wolkoff, A.W. and Chung, C.T. (1980) J. Clin. Invest. 65. 1152-1161.

Wood, G.C. and Cooper, P.F. (1970) Chromatog. Rev. 12, 88-107.

REFERENCES ADDED AFTER BINDING

van Dyke, R.W., Stephens, J.E. and Scharschmidt, B.F. (1982) Am. J. Physiol. 243, G484-492.

Suchy, F.J., Garfield, S.A., Balistreri, W.F. and Miller, P. (1982) Gastroenterology 82, 1246.

Suchy, F.J., Balistreri, W.F., Hung, J., Miller, P. and Garfield, S.A. (1983) Am. J. Physiol. 245, G681-689.

PUBLICATIONS

The following paper was published as a result of work presented in this thesis.

Biochimica et Biophysica Acta, 795 (1984) 257–264
Elsevier

BBA 51739

SYNTHESIS AND CHARACTERISATION OF AN IODINATED BILE-SALT DERIVATIVE FOR PHOTOAFFINITY LABELLING

COLIN J. HENDERSON and IAIN W. PERCY-ROBB

University Department of Clinical Chemistry, The Royal Infirmary, Edinburgh EH3 9YW (U.K.)

(Received March 14th, 1984)

Key words: Bile salt; Photoaffinity labelling; (Rat liver)

The synthesis and characterisation of a novel iodinated bile salt derivative, ^{125}I-labelled 3β-azidocholylhistamine, is described. The derivative is handled by rat liver in a similar manner to taurocholate and binding to bovine serum albumin, a well-characterised bile acid-binding protein, is demonstrated. The suitability of the derivative for photoaffinity labelling is assessed.

Introduction

The enterohepatic circulation describes the recycling of bile salts between the intestine and the liver. The transport systems involved in carrying the bile salts across the intestinal epithelium [1,2] and from the sinusoidal border of the hepatocytes to the canaliculi [3–5] have been partially characterised. Bile salt-binding proteins have been demonstrated in both the intestinal mucosa [6] and hepatocyte sinusoidal and canalicular membranes [7–11].

One potential transcellular route for bile salts is in association with cytosolic proteins [12]. Three main groups of bile salt-binding proteins have so far been described. The glutathione *S*-transferases (EC 2.5.1.18) have received particular attention [13,14]; these enzymes have a molecular mass of approx. 45 kDa and are abundant in the hepatocytes, making up 3–5% of the total cytosolic protein. Bile salt binding to proteins with a molecular mass of approx. 12 kDa, which has been called Z protein [15], fatty acid-binding protein [16], aminoazodye-binding protein A [17], squalene and sterol carrier protein [18], has been recognised for a number of years. A newly discovered group, termed Y′ (molecular mass approx. 35–40 kDa), has also been proposed as a component of the bile salt transport system [19].

The bile acid-binding properties of these proteins have been demonstrated using reversible techniques. However, experiments to identify the polypeptides responsible for binding and the structures of the binding sites themselves require the formation of stable bile acid-protein complexes which are amenable to further study using procedures such as proteolytic digestion and peptide analysis. Such ligand-protein complexes could be achieved by using the technique of photoaffinity labelling. Although the first experiments in this area were performed over 20 years ago [20], recent reviews testify to the expanding nature of this field [21,22]. The method employs a photolytically generated intermediate of the ligand, which has very high reactivity and which will covalently attach to the ligand-binding site of the protein.

The synthesis of bile acid-derived photoaffinity probes has been described [23,24]. In each case, these have been radiolabelled using beta-emitting isotopes, in most the specific activity being low (1–3 Ci/mmol). To be able to work at the con-

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, *p*-bis(2-(5-phenyloxazolyl))benzene; ACH, 3β-azidocholylhistamine.

centration of free bile acid in the hepatocyte, estimated to be in the nmol or pmol range, and subsequently to analyse the binding, it is necessary to use a photolabile bile acid with a high specific radioactivity. We now describe the synthesis of a novel bile salt derivative, ^{125}I-labelled 3β-azidocholylhistamine, which is radioiodinated for high specific activity (approx. 1900 Ci/mmol); gamma labelling of the derivative improves the sensitivity and convenience of detecting and counting the radioactive polypeptides. The synthesis, characterisation, hepatic transport and covalent linking to albumin of the derivative are described.

Materials

All chemicals were purchased from either Macfarlane Robson Ltd., Glasgow, U.K. (BDH agents) or Sigma Chemical Co. (London) Ltd., Poole, Dorset, U.K., and were of analytical grade. Toluene used for scintillation counting was scintillation grade. Cholic acid was recrystallised four times from methanol. Sodium [^{125}I]iodide (approx. 1900 Ci/mmol) and tauro[*carbonyl*-^{14}C]cholic acid, sodium salt (60.9 mCi/mmol), were from Amersham International, Amersham, Bucks, U.K., and 2,4-[^{3}H]cholic acid (3 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A. All bile salts were shown to be at least 99% pure on TLC. Pyridine was dried by refluxing with potassium hydroxide pellets for 2 h and collected by distillation at 114°C. The *p*-toluenesulphonyl chloride was recrystallised hot from petroleum ether (40–60°C) as white monoclinic crystals. Dimethyl sulphoxide was dried by refluxing with sodium hydroxide pellets for 30 min and collected by distillation at 189°C.

Methods

Characterisation of bile acid derivatives. Ultraviolet scans were performed on a Pye-Unicam SP-1800 double-beam spectrophotometer and infrared scans on a Pye-Unicam SP-200 double-beam spectrophotometer (Pye-Unicam Ltd., Cambridge, U.K.). Melting points were determined with a Reichert Thermopan Microscope (C Reichert, Optische Werke AGm, Wien, Austria). ^{1}H- and ^{13}C-NMR spectra were measured on a Bruker 200 MH_3 instrument (Bruker, Zurich, Switzerland). The solvent for ^{1}H scans was deuterated chloroform, (C^2HCl_3) and for ^{13}C, dimethyl sulphoxide-d_6. ^{125}I was measured in a Nuclear Enterprises NE16 gamma counter (Nuclear Enterprises, Sighthill Industrial Estate, Edinburgh, U.K.) and ^{14}C was counted in a Packard A3255 scintillation counter (Technologies Packard, Caversham, Bucks, U.K.) using a toluene-based scintillant (6 g PPO, 0.3 g POPOP, 1 l toluene, 0.5 l Triton X-100).

Photolysis. Photolysis experiments were carried out at 37°C in an annular photoreactor (Applied Photophysics Ltd., London, U.K.) with a 400 W medium-pressure mercury arc lamp having an output of more than $5 \cdot 10^{19}$ photons/s and radiating predominantly at 313 nm and 365 nm, using a borosilicate glass filter with wavelength cut-off of approx. 300 nm. Test solutions (10 ml) were held in quartz tubes (11 mm i.d.) with a glass rod (7 mm o.d.) located centrally to give a thin liquid film of approx. 2 mm. The tubes were rotated at 25 rpm around the light source.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [25] using Raven apparatus (Raven Scientific, Haverhill, Suffolk, U.K.) with a 3.6% stacking and 7.5% resolving gel (195 × 40 × 1.5 mm).

Chromatography. Gel filtration was performed at 4°C on columns (2.6 × 42 cm) of Sephadex G-100 fine which were eluted with 0.1 M sodium phosphate (pH 7.4) (buffer A). Fractions of 7 ml were collected.

Thin-layer chromatography was performed on silica gel 60 plates (5 × 20 cm) and silica gel column chromatography in glass columns (2.5 × 90 cm) using silica gel N-60 (43–60 μM), both from Merck (Merck, Darmstadt, F.R.G.). The following solvent systems were used: 1, ethyl acetate/cyclohexane/acetic acid (23 : 7 : 3 v/v); 2, ethyl acetate/cyclohexane/acetic acid (100 : 40 : 1 v/v); 3, trimethylpentane/isopropyl alcohol/acetic acid (120 : 40 : 1 v/v); 4, chloroform/methanol/acetic acid/water (30 : 10 : 3 : 2 v/v).

Bile acids were visualised on the TLC plates with a freshly prepared solution of glacial acetic acid/sulphuric acid/anisaldehyde (100 : 2 : 1 v/v).

Synthesis of ^{125}I-labelled 3β-azidocholylhistamine [23]. A solution of *p*-toluenesulphonyl chloride (5

mmol) in absolute pyridine (2 ml) was added (over 30 min, 0 °C with stirring) to a solution of cholic acid (5 mmol) in absolute pyridine (10 ml), also containing 2,4-[^{3}H]cholic acid (0.1 μCi, 65 μmol) as tracer. The solution was warmed and stored at room temperature for 3 h, the progress of the reaction being monitored by TLC in solvent system 1. When complete, the reaction mixture was added slowly, with stirring, to 1.5 M hydrochloric acid (400 ml) and the precipitate collected by filtration and dried in vacuo over silica crystals. This material was dissolved in solvent system 1 and applied to a 2.5 × 70 cm column of silica gel, developed with solvent system 1; 1.038 g of 3α-toluenesulphonyl cholic acid was recovered (1.85 mmol, 40% yield).

The 3α-toluenesulphonyl cholic acid (1.85 mmol) was dissolved with stirring in a warm solution of sodium azide (15.5 mol) in dry dimethyl sulphoxide (23 ml) and the resulting solution was kept in the dark at 100 °C for approx. 5 h. All further work was carried out in dim or red light. The progress of the reaction was monitored by TLC in solvent system 1 and, when complete, the solution was poured with stirring into 1 M hydrochloric acid (500 ml). The resulting precipitate was collected by filtration and dried in vacuo over silica crystals. The products obtained were purified by chromatography on a 2.5 × 70 cm column of silica gel, eluted using solvent system 2, yielding 300 mg of 3β-azidocholic acid (0.7 mmol, 40% yield).

Addition of histamine and subsequent iodination of 3β-azidocholic acid was carried out according to Beckett et al. [26], TLC in solvent system 4 being used to separate iodinated from non-iodinated conjugate in order to maximise the specific radioactivity of the final product. ^{125}I-labelled ACH was eluted from the silica gel by ethanol and stored in the dark at 4 °C. The yield was 25 nmol of ^{125}I-ACH with a specific radioactivity of approx. 1900 Ci/mmol. The iodination procedure was used on a 10^3-times larger scale with unlabelled sodium iodide to synthesise iodo-ACH for infrared spectroscopy. The structures of the compounds involved in the synthesis are shown in Fig. 1.

Stability of ^{125}I-ACH in ultraviolet light. ^{125}I-ACH (10 μCi) in methanol (10 ml) was photolysed for varying times (0–60 min) both with and without the borosilicate glass filter. At 0, 5, 10, 20 and 30 min, portions (50 μl) were removed and spotted on to silica gel TLC plates (5 × 20 cm) and developed in solvent system 4. After drying, the silica gel was divided into 1 cm squares which were scraped into vials before counting in a gamma counter.

Stability of bovine serum albumin in ultra-violet light. Bovine serum albumin (98–99%, essentially fatty acid-free) had endogenous bile acids removed by the method of charcoal-stripping and was shown to be bile acid-free by radioimmunoassay [27]. Portions of bovine serum albumin (100 mg) in buffer A (10 ml) were photolysed both with and without the glass filter. Portions (50 μl) were removed from these solutions at timed intervals and analysed by SDS-electrophoresis.

Photolysis of ^{125}I-ACH with bovine serum albumin. Bovine serum albumin (98–99%, essentially fatty acid free) had endogenous bile acids removed by the method of charcoal-stripping and was shown to be bile acid-free by radioimmunoassay [27]. Portions of bovine serum albumin (100 mg) in buffer A (10 ml) were photolysed in the presence of ^{125}I-ACH (10 μCi, 5 pmol), both with and without the glass filter. Portions (50 μl) were removed from these solutions at timed intervals and analysed by SDS-electrophoresis. After staining, the gel tracks were cut out, sliced into 0.5 cm pieces and the amount of ^{125}I-ACH associated with each was determined. In addition, the 60 min sample in each case was applied to a column of Sephadex G-100 fine (2.6 × 42 cm).

Hepatic transport of ^{125}I-ACH. Wistar rats, 150–250 g, fed ad libitium, were anaesthetised with diethyl ether and the common bile duct was cannulated with nylon tubing (i.d. 0.1 mm). The test solutions were injected into the superior mesenteric vein in 150 mM saline (75 μl) containing approx. 20% ethanol (v/v). Bile was collected at 1 min intervals in pre-weighed tubes and the radioactivity counted. For detection of the ^{125}I-labelled derivative, the bile samples were counted directly, whereas ^{14}C-labelled bile acids were counted following the addition of scintillant (5 ml).

Results

The structures of the starting material (A) and intermediate products (B–D) and ^{125}I-ACH (E) are shown in Fig. 1. The formation of the 3α-toluenesulphonyl derivative of cholic acid was demonstrated on TLC and confirmed by infrared spectroscopy. A characteristic peak at 1705 cm^{-1} confirmed that the saturated ketone (C = O) from the carboxylic side-chain of the bile acid was still present. Formation of the 3β-azido derivative of cholic acid was also demonstrated by a change in the mobility of the substrate on TLC. Infrared spectroscopy confirmed the incorporation of the photolabile azido (N_3) group by virtue of the 2105 cm^{-1} peak, and also showed the retention of the 1705 cm^{-1} peak for the bile acid side-chain. The data from TLC mobilities, melting points and ultraviolet scan were all consistent with those of Kurz and Gerok [23]. The NMR spectra confirmed the final structure of the molecule.

The third stage of the synthesis involves the histamination and subsequent iodination of the 3β-azidocholic acid, shown on TLC, and the azido (N_3) group demonstrated on infrared.

Stability of ^{125}I-ACH. The stability of the photolabile derivative during photolysis was assessed. A solution of ^{125}I-ACH was exposed to ultraviolet light both with and without the borosilicate glass filter which provided a wavelength cut-off at approx. 300 nm. In both cases the photolysis led to a loss of radioactivity from the mobility (on TLC) of

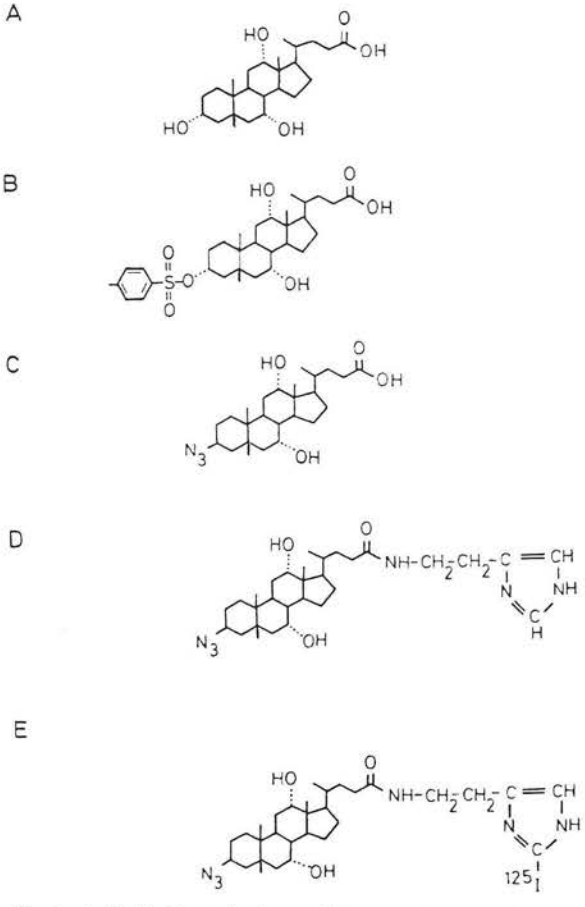


Fig. 1. A, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (cholic acid); B, 3α-toluenesulphonyl cholic acid; C, 3β-azidocholic acid; D, 3β-azidocholylhistamine; E, ^{125}I-labelled 3β-azidocholylhistamine.

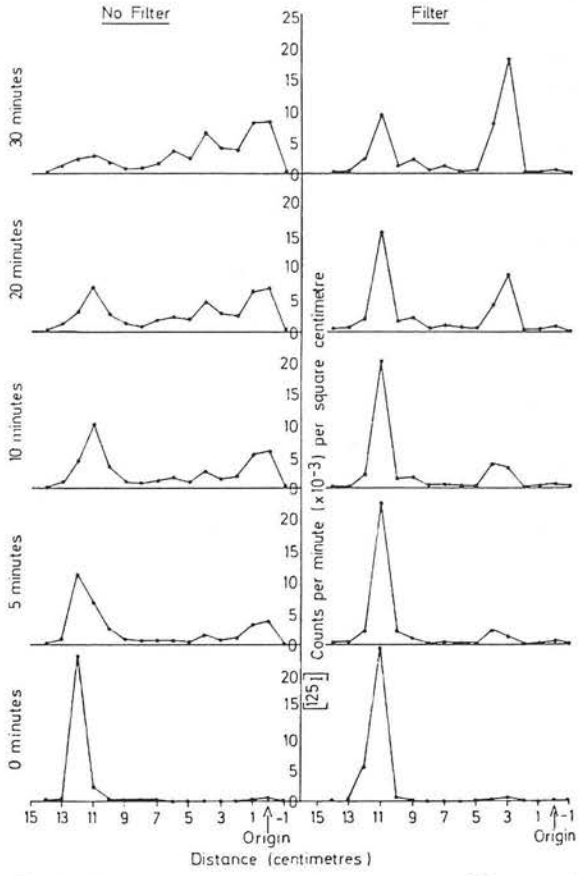


Fig. 2. Photolysis, with and without filter, of ^{125}I-labelled 3β-azidocholylhistamine (5 pmol, 10 μCi) in buffer A. Samples were run on TLC, solvent system 4.

the derivative (R_F 0.79) and an increase of radioactivity at the origin or at a point close to but clearly different from the origin (R_F 0.21, see Fig. 2). With no filter in position, the loss of radioactivity was rapid, the majority of the radioactivity appearing at the origin. This is consistent with the mobility of free iodide. About 20% of the radioactivity appeared at a position corresponding to that of iodinated histamine (R_F 0.2). With the filter in position, the loss of radioactivity was slower; approx. 40% of the radioactivity was still at the position of the original bile acid derivative after 30 min. In this case, no free iodide was detected, all of the released radioactivity being at a position consistent with that of iodinated histamine (R_F 0.2).

Stability of protein during photolysis. When bile acid-free bovine serum albumin was photolysed under similar conditions, light below 300 nm proved to be more destructive. On SDS-electrophoresis, less than 10% of the bovine serum albumin retained the correct electrophoretic mobility after 60 min photolysis without the filter, whereas more than 90% remained with the filter in place (Fig. 3).

Photolysis of ^{125}I-ACH with bovine serum albumin. A solution of bile acid-free bovine serum albumin incubated with ^{125}I-ACH which was photolysed both with and without the filter for 60 min was analysed by gel filtration (Fig. 4A and B). In the presence of the filter, the ^{125}I label was associated with the bovine serum albumin peak (M_r approx. 70 kDa), and unreacted label was found in the salt volume of the column ($M_r < 1000$ Da). However, without the filter, the radioactivity was found with a higher molecular mass peak (M_r 150

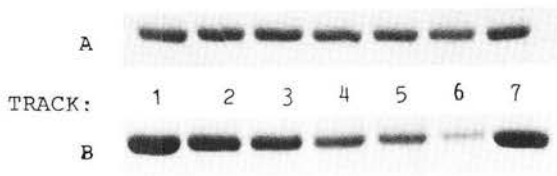


Fig. 3. Photolysis, with (A) and without (B) filter, of bile acid-free bovine serum albumin in buffer A. Tracks 1 and 7, control (unphotolysed) bovine serum albumin. Tracks 2–6, bovine serum albumin photolysed for 5, 10, 20, 30 and 60 min, respectively.

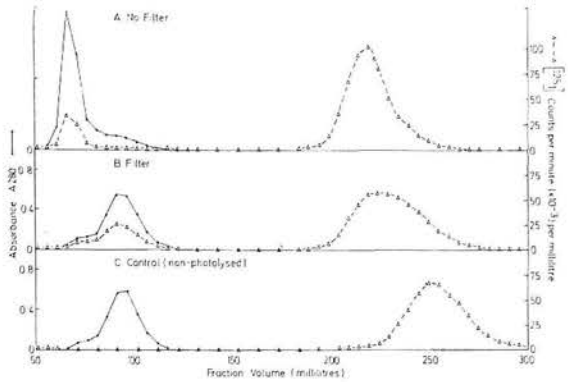


Fig. 4. Gel filtration on Sephadex G-100 fine of photolysed (60 min, no filter and filter) bovine serum albumin samples (A and B) and control (C). Bile acid-free bovine serum albumin (5 ml, 10 mg/ml) and ^{125}I-labelled 3β-azidocholylhistamine (2.5 pmol 5 μCi). Absorbance A_{280}, ●——●; ^{125}I-ACH, ◁— — —▷.

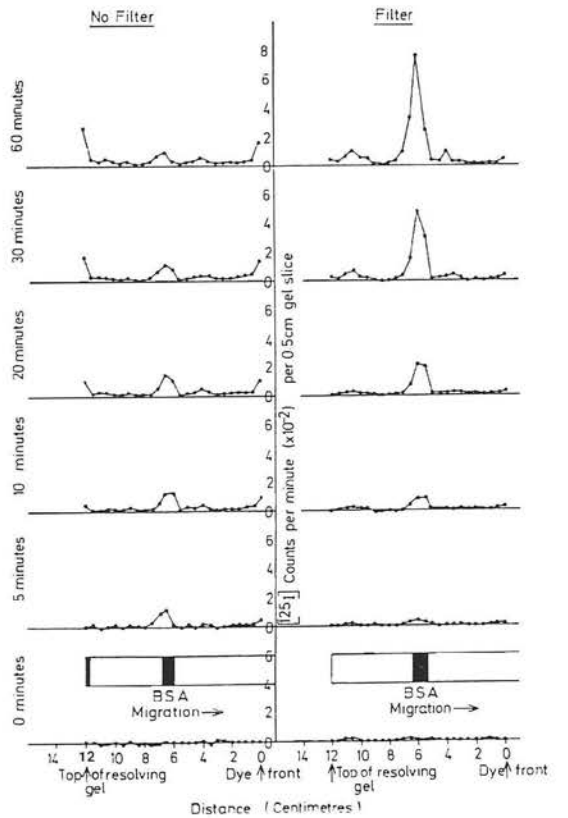


Fig. 5. SDS-electrophoresis of photolysis (filter and no filter) samples and control. Bile acid-free bovine serum albumin (10 ml, 10 mg/ml) and ^{125}I-labelled 3β-azidocholylhistamine (5 pmol 10 μCi). Insets show bovine serum albumin band after 60 min photolysis.

kDa), the result of photolytic-mediated aggregation of the protein. Again, unreacted label was found in the salt volume of the column. A mixture of bovine serum albumin and ^{125}I-ACH was passed down the column (Fig. 4C) without prior photolysis, and served as a control.

The labelling of bovine serum albumin with ^{125}I-ACH was also investigated by SDS-electrophoresis. Fig. 5 shows the incorporation of ^{125}I into the bovine serum albumin band after photolysis both with and without the filter for times between 0 and 60 min. Labelling of bovine serum albumin was greater when the filter was used. With no filter, ^{125}I was found in high and low molecular weight bands at the top and bottom of the gel, respectively.

Hepatic transport of ^{125}I-ACH. ^{125}I-ACH and ^{14}C-labelled taurocholate were injected simultaneously into the superior mesenteric vein following bile duct cannulation. For both bile acids, radioactivity first appeared after approx. 3 min (Fig. 6). However, although the maximum of ^{14}C-labelled taurocholate excretion was slightly earlier (6 min) than that of ^{125}I-ACH (8 min), the overall excretion profiles of the two compounds were very similar.

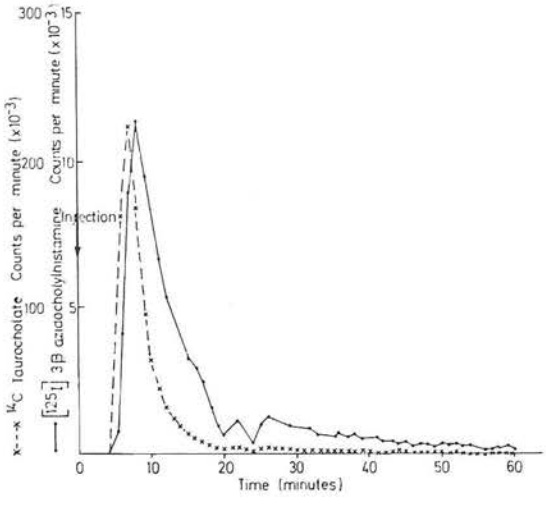


Fig. 6. Biliary excretion of ^{125}I-labelled 3β-azidocholyl histamine and ^{14}C-labelled taurocholate after injection (125 fmol, 0.25 μCi and 0.8 nmol, 0.5 μCi, respectively) in 154 mM saline (75 μl) with 15% ethanol (v/v) into the superior mesenteric vein. ^{14}C, ×— — —×; ^{125}I, ●———●.

Discussion

The introduction of a photolabile azido group into the cholic acid molecule has resulted in a compound with a wide range of applications as a photoaffinity probe. Conjugation of this molecule with histamine and subsequent iodination yields the high specific radioactivity (approx. 1900 Ci/mmol) necessary to investigate bile acid-binding to cytosolic proteins in the liver.

It is essential that this compound is handled by the liver in a similar manner to physiological bile salts in order to be able to relate experimental results with the photoaffinity probe to events occurring within the hepatocyte. The studies with rat liver demonstrate that ^{125}I-labelled 3β-azidocholylhistamine is cleared from the liver and excreted into bile in a manner which closely resembles that of the natural bile salt, taurocholate. A similar molecule, ^{131}I-, ^{125}I- or ^{123}I-cholylglycylhistamine has been used by several groups [28–30] in experiments to demonstrate hepatic uptake and excretion and to show the existence of a hepatic concentration gradient for bile acid uptake in rats. In each case, hepatic handling of the bile salt derivative was similar to physiological bile salts. Indeed, Grandjean et al. [30] have suggested that the side-chain of a bile acid molecule need not possess an anionic charge in order to be taken up and excreted by the liver, as had previously been proposed for intestinal transport [1].

The results of the study in which ^{125}I-ACH was photolysed alone showed that the wavelength of light used is important. With light below 300 nm, destruction of the derivative was rapid, resulting mainly in the release of free iodide, but also, to a smaller extent, in the release of iodinated histamine, due to breakage of the internal peptide bond of the derivative between the steroid ring structure and the histamyl moiety. However, with light above 300 nm, destruction of the derivative was much slower, the breakdown occurring by release of iodinated histamine only. This information, together with the results of the test photolysis of bile acid-free bovine serum albumin with ^{125}I-ACH, would suggest that the derivative can be employed as a photoaffinity label, and that irradiation should be carried out, as it has been by other groups [6,11,24], with light above 300 nm. However, the

TABLE I

Molecule	Melting point (°C)	Thin-layer chromatography [a]	Ultraviolet λ_{max}	Ultraviolet ε	Infrared	NMR ^1H	NMR ^{13}C
Cholic acid		0.19/0.02			1705 cm^{-1} (C = O)		
3α-Toluenesulphonyl cholic acid		0.86/0.54			1705 cm^{-1} (C = O)		
3β-Azidocholic acid	209–212	0.85/0.39	220 nm 285 nm	262 45	2105 cm^{-1} (N_3) 1700 cm^{-1} (C = O)	C^2HCl_3 δ = 0.7 (S, CH_3 – 18) 0.92 (S, CH_3 – 19) 0.97 (*d*, *J* = 6Hz, CH_3 – 21)	DMSO-d_6 δ = 174.9 (CO_2H) Total = 24 C atoms C = 3 CH = 9 CH_2 = 9 CH_3 = 3 24
^{125}I-labelled 3β-azidocholyl histamine		0.68/0.82			2100 cm^{-1} (N_3) 1570 cm^{-2}		

[a] Data are R_F values in solvent system 1/solvent system 2.

maxima of ultraviolet absorption of the derivative are at 220 and 285 nm (Table I) and the most efficient photolysis should be affected at these wavelengths.

The photolysis was more successful with light above 300 nm, as judged by the greater incorporation of radioactivity observed in the bovine serum albumin band on SDS-electrophoresis (Fig. 5). However, using light below 300 nm, the bovine serum albumin was broken down more rapidly, which may partly explain the lower incorporation of ^{125}I. The fact that photolysis has occurred at all, when the ultraviolet absorption maxima of the derivative lie below 300 nm, is probably due to the absorption of light by the molecule at the long-wavelength tail of its 285 nm maximum, which is sufficient to stimulate the photolabile azido group. This has been discussed in more detail recently [31].

These studies have shown that the new derivative of cholic acid, with very high specific radioactivity, is suitable for photoaffinity labelling studies, being linked covalently to protein. The derivative is also handled effectively as a bile salt by the liver. Experiments with ^{125}I-ACH using rat liver cytosolic proteins are being performed.

Acknowledgements

Colin J. Henderson thanks the University of Edinburgh for a Faculty of Medicine Scholarship. We thank Dr. I. Gosney of the Chemistry Department, University of Edinburgh for ^{1}H and ^{13}C NMR spectra and Dr. J.E.T. Corrie, Teaching and Research Unit, Western General Hospital, Edinburgh, for advice. We are greatly indebted to Professor G. Kurz, Chemisches Laboratorium der Universtat Freiburg, D-7800, Freiburg, F.R.G., for generously sending us a copy of his paper before its publication. We also thank Professor L.G. Whitby and Dr. J.D. Hayes for critically reading this manuscript and Mrs. E. Ward and Mrs. C. Veitch for their secretarial assistance.

References

1 Wilson, F.A. and Treanor, L.L. (1975) Biochim. Biophys. Acta 406, 280–293
2 Wilson, F.A. (1981) Am. J. Physiol. 241, G83–92
3 Van Dyke, R.W., Stephens, J.E. and Scharschmidt, B.F. (1982) Am. J. Physiol. 243, G484–492
4 Reichen, J. and Paumgartner, G. (1976) Am. J. Physiol. 231, 734–742
5 Blitzer, B.L., Rataash, S.I., Donovan, C.B. and Boyer, J.L. (1982) Am. J. Physiol. 243, G48–53
6 Kramer, W., Burckhardt, G., Wilson, F.A. and Kurz, G. (1983) J. Biol. Chem. 258, 3623–3627

7 Anwer, M.S., Kroker, R., Hegner, D. and Petter, A. (1978) Hoppe-Seyler's Z. Physiol. Chem. 358, 543–553
8 Anwer, M.S., Kroker, R., and Hegner, D. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1477–1486
9 Accatino, L. and Simon, F.R. (1976) J. Clin. Invest. 57, 496–508
10 Ohkima, S. and Kuriyama, K. (1982) Steroids 39, 7–21
11 Kramer, W., Bickel, U., Buscher, H.P., Gerok, W. and Kurz, G. (1982) Eur. J. Biochem. 129, 13–24
12 Strange, R.C. (1981) Hepatic bile salt transport. Biochem. Soc. Trans. 9, 170–174
13 Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1979) Biochem. J. 181, 699–708
14 Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1980) Biochem. J. 185, 83–87
15 Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1969) J. Clin. Invest. 48, 2156–2167
16 Ockner, R.K., Manning, J.A., Poppenhausen, R.B. and Ho, W.K.L. (1972) Science 177, 56–88
17 Ketterer, B., Tipping, E., Hackney, J.F. and Beale, D. (1976) Biochem. J. 155, 511–521
18 Dempsey, M.E., McCoy, K.E., Baker, H.N., Dmitriadou-Vafiadou, A., Lorsbach, T. and Howard, J.B. (1981) J. Biol. Chem. 256, 1867–1873
19 Sugiyama, Y., Yamada, T. and Kaplowitz, N. (1983) J. Biol. Chem. 258, 3602–3607
20 Singh, A., Thornton, E.R. and Westheimer, F.H. (1962) J. Biol. Chem. 237, 3006–8
21 Chowdhry, V. (1979) Annu. Rev. Biochem. 48, 293–325
22 Bayley, H. and Knowles, J.R. (1977) Methods Enzymol. 46, 69–114
23 Kurz, G. and Gerok, W. (1983) J. Lip. Res. 24, 910–923
24 Von Dippe, P., Drain, P. and Levy, D. (1983) J. Biol. Chem. 258, 8890–8895
25 Laemmli, U.K. (1970) Nature (London) 227, 680–685
26 Beckett, G.J., Corrie, J.E.T. and Percy-Robb, I.W. (1979) Clin. Chim. Acta 93, 145–150
27 Beckett, G.J., Hunter, W.M. and Percy-Robb, I.W. (1978) Clin. Chim. Acta 88, 257–266
28 Spenny, G.J., Tobin, M.M., Mihas, A.A., Gibson, R.G., Hirschowitz, B.I., Johnson, B.J. and Tauxe, W.N. (1978) Gastroenterology 76, 272–278
29 Jones, A.L., Hradek, G.T., Renston, R.H., Wong, K.Y., Karlaganis, G. and Paumgartner, G. (1980) Am. J. Physiol. 238, G233–237
30 Grandjean, E.M., Karlaganis, G., Noelpp, U., Hwegli, H., Roesler, H. and Paumgartner, G. (1978) in The Liver-3rd International Gstaad Symposium (Preisig, R. and Paumgartner, G., eds.), Edition Cantor, pp. 255–262, Aulendorf, F.R.G.
31 Nielsen, P.E., Hansen, J.B., Thomsen, T. and Buchardt, O. (1983) Experientia 39, 1063–1072